VOLUME 25 ISSUE 1 February 2020 ISSN: 1744-165X
www.sfnmjournal.com

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FETAL & NEONATAL
medicine

BONE METABOLIC DISORDERS IN PREMATURE AND FULL TERM NEONATES

Guest Editor: Charalampos Dokos

Seminars in Fetal and Neonatal Medicine 25 (2020) 101089

Contents lists available at ScienceDirect

Seminars in Fetal and Neonatal Medicine

journal homepage: www.elsevier.com/locate/siny

Editorial

Bone metabolic disorders in premature and full term neonates: Current status and future directions

Bone metabolic disorders are a unique area of research in neonatology. In the latest decade there is a great interesting concerning the consequences of prematurity and bone metabolism. Premature infants seem to be a high risk group of newborns with a great incidence to develop bone metabolic disorders. Metabolic bone disease (MBD) of prematurity is a multifactorial disorder that is observed in very low birth weight newborns. MBD of prematurity has a greater incidence in those extremely low birth weight infants. It is characterized by biochemical and radiological findings related to bone demineralization. As a result of this skeletal demineralization are distinct fragility fractures of long bone and ribs.

The latest trimester is the most important period of a pregnant, during that there is an intensive fetal mineral accumulation. Several prenatal and postnatal factors have been identified although the pathogenetic mechanisms that involve placenta are not clarified. In the field of diagnostics, screening and therapeutics, there is no consensus among neonatologists and societies. This is an exciting opportunity to investigate in depth the MBD of prematurity with the use of biomarkers of bone metabolism, radiological findings and ultrasonographic devices. The treatment is determined from the aetiology of MBD, therefore we should be very careful to optimize the mineral imbalance and validate the progress of the disease.

In this issue it is a pleasure to have leading researchers worldwide to cover a range of topics concerning MBD in neonates. Schulz and Wagner from the Medical University of South Carolina give us a brief review of the history, epidemiology and prevalence of neonatal MBD. Through the evolution of mankind and human migration combined with cultural, lifestyle and religious influences the prevalence of MBD in neonates was changed. Sethi, Priyadarshi and Agarwal discuss the mineral and bone physiology in fetus, preterm and full-term neonates. Ryan and Kovacs from the Memorial University of Newfoundland review a very special theme concerning the bone and mineral metabolism, the role of the calciotropic and phosphotropic hormones in fetal and neonatal bone development. El-Farrash from Ain Shams University of Egypt introduces us the post-natal bone physiology.

Ramon's review examines the risk factors concerning bone mineral metabolism disorders in neonates. Rayannavar and Calabria give us a state of art review about the screening and clinical diagnosis of neonatal MBD. The authors explain the screening and clinical tools (biochemical, imaging) to identify high risk neonates. It seems that preterm infants require specific mineral and nutritional support, therefore Professor Czech-Kowalska from the Medical University of Warsaw explain the different therapeutic approaches and clinical pearls in this area. In the quest for novel therapeutic intervention in neonatal MBD, Chinoy, Zulf Mughal and Padidela from the Royal Manchester Children's Hospital introduce us in this brief review the current status in neonatal bone mineraltherapeutics.

There is a great interest concerning the MBD in premature and full-term neonates. The authors of this issue have approached many of the aspects of this area but also point to the future research directions. The clinical expertise and experience are important in the therapeutical management of neonatal MBD. Optimization of feeding protocols and parenteral nutrition guidelines are vital in the prevention and treatment of high risk neonates. Also there is mounting evidence of the strong molecular and biochemical background of bone health regulation in premature neonates.

Charalampos Dokos
2nd Neonatal Clinic, Papageorgiou University Hospital, Medical School, Aristotle University of Thessaloniki, Greece

https://doi.org/10.1016/j.siny.2020.101089

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Seminars in Fetal and Neonatal Medicine 25 (2020) 101069

Contents lists available at ScienceDirect

Seminars in Fetal and Neonatal Medicine

journal homepage: www.elsevier.com/locate/siny

History, epidemiology and prevalence of neonatal bone mineral metabolic disorders

Elizabeth V. Schulz*, Carol L. Wagner

Department of Pediatrics, Medical University of South Carolina, 165 Ashley Avenue, MSC 917, Charleston, SC, 29425, USA

ARTICLE INFO

Keywords:
Infant
Bone
Rickets
Vitamin D
Parenteral nutrition

ABSTRACT

The evolutionary patterns of human migration and historical pre/post-industrial revolution have changed the face of bone metabolic disease through past centuries. Cultural, religious, and lifestyle practices continue to alter nutritional recommendations for this expanding diagnosis. Likewise, modern advancements in the field of neonatology and, more specifically, aggressive nutritional management of premature infants have shaped the epidemiology of neonatal bone metabolism over the past two decades. Decreased use of long-term parenteral nutrition, early fortification of enteral nutrition, and stringent American Academy of Pediatrics (AAP) practice guidelines instituting early supplementation of vitamin D have attributed to improved bone mineralization outcomes in both term and preterm infants. Nevertheless, neonatal bone mineral metabolic disorders remain prevalent. In this review, we provide an in-depth look at the diagnoses, therapeutics, and subset populations—both genetic and non-genetic—affected by neonatal bone mineral metabolic disorders.

1. Introduction

Advancements in the field of neonatology and, more specifically, aggressive nutritional management of premature infants have shaped the epidemiology of neonatal bone metabolism over the past two decades. Decreased use of long-term parenteral nutrition, early fortification of enteral nutrition, and stringent American Academy of Pediatrics (AAP) practice guidelines instituting early supplementation of vitamin D have attributed to improved bone mineralization outcomes in both term and preterm infants. Nevertheless, neonatal bone mineral metabolic disorders remain prevalent, especially in very low birth weight (VLBW, < 1500 g) and extremely low birth weight (ELBW, < 1000 g) infants, as well as infants born at lower gestational ages [1–3]. In this review, we provide an in-depth look at the origins of neonatal metabolic bone disorders as well as the diagnoses, therapeutics, and subset populations—both genetic and non-genetic—affected by such disorders.

2. Historical, geographic, and ethnic epidemiologic perspectives on metabolic bone disease

From phytoplankton fossils to modern day mammals, the capacity to make and utilize an active form of vitamin D is both historical and universal. The role of vitamin D in bone development is widely accepted. Nevertheless, the epidemiology of metabolic bone disease continues to change through the generations and throughout the world. The human geographic migration as well as customs and traditions have altered scientific outlook on the diagnosis and treatment of nutritional rickets. Dubbed the "vitamin D hypothesis", the refinement of the original descriptions of human skin pigmentation and the vitamin D receptor (VDR) following migration and settlement within an array of latitudes has progressed through the 20th century [4]. Evolutionary analyses have demonstrated the ancestral need for dark skin as a trait in regions of high UV intensity [5]. This pigmentation, as exhibited by reflectance measurements, is due to the quantity, size, and distribution of melanin. The increased cutaneous melanin slows synthesis of vitamin D_3, making this an evolutionary adaptation to regulate synthesis of vitamin D_3 depending on the quantity of pigmentation a human has or needs. Migrational patterns to northern latitudes and the subsequent epidemiologic quantitative loss of melanin corroborates this evolutionary standard [6].

Following the depigmentation effects of polar migrations out of equatorial tropics, Hochberg cites the agrarian revolution as the next change in vitamin D metabolism for humans [4]. Although the primal need to reproduce our own DNA, to include phenotypic propagation, persists throughout time, knowledge and skills are similarly passed

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[*] Corresponding author. Medical University of South Carolina, Department of Pediatrics, Division of Neonatology, 165 Ashley Avenue, MSC 917, Charleston, SC, 29425, USA.

E-mail addresses: eschulz033107@gmail.com (E.V. Schulz), wagnercl@musc.edu (C.L. Wagner).

https://doi.org/10.1016/j.siny.2019.101069

1744-165X/ Published by Elsevier Ltd.

through the generations. Within the agrarian society, humans learned to domesticate dairy animals providing a steady source of food and calcium. It is within these anthropologic changes that the first reports of nutrition as a healing power for disease would emerge, but it would not be until the 19th century that these remedies would be scientifically founded.

Food, shelter, and clothing are the cornerstones of basic human needs and as such, they are woven in the story of human vitamin D and VDR adaptations. Following the agrarian revolution, it would be centuries before the scientific and industrial revolutions further altered the epidemiology of bone metabolic disease. Nevertheless, a migration to city dwelling, indoor (vs farm) work, and technology led to whole population deficits in sunlight, thus increasing the incidence of vitamin D deficiency and rickets. Referred to historically as “the English disease”, rickets was overwhelmingly present in Great Britain during the late 18th and early 19th centuries due to overcrowding, which led to starvation, as well as industrial air pollution, quite literally limiting sunlight [4]. It is within this timeline that classical nutritional discoveries graced scientific literature. Estimated to be present in 50–80% of northern European children in the 19th century, rickets (along with tuberculosis) was a prevalent diagnosis [4,7]. However, the introduction of cod liver oil, which contains vitamin D, notably improved the clinical outcomes of patients with consumption [4]. This precursory treatment has been the foundational approach to vitamin D supplementation for a variety of illnesses, even in modern times. It is also within the industrial revolution that beloved fictional characters, with suspected rickets, rose to popularity. The descriptive narration of Tiny Tim, from Charles Dickens' novel “A Christmas Tale” (c. 1843), won the hearts of the world as a crippled boy with a positive attitude. It is posited Dickens created the character in reflection of the numerous children on the streets of London, at that time, with phenotypic evidence of nutritional deficiencies and rickets. The character, described as having a “short stature, asymmetric crippling, and curious intermittent weakness that would lead to his death, if untreated” [7], has received a variety of diagnoses throughout the last two centuries—all of which describe either a genetic or nutritional etiology to his suspected bone ailments. Some critics feel the character's debilitations were born of dietary restrictions related to poverty during the era. Others suggest his osteopenic suffering was from a renal tubular acidosis (RTA) variant (likely distal-type RTA), causing bone resorption and hypercalciuria, buffering the metabolic acidosis seen in this disorder. As a result, the character experienced weakened bones and lower extremities, leading to a plausible diagnosis of rickets. It is within these pieces of documentation, both scientific and fictional, that medical and legal advancements would change the face of vitamin D deficiency for the next generation.

With the advent of clean air legislation in Europe and nutritional supplementation throughout affluent countries, a paradigm shift occurred in the evolution of human metabolic bone disease. A resurgence of the clinical presentations of rickets has presented in Westernized countries, which has been proposed to be the result of sun counseling and UV protection. Nevertheless, studies continue to reveal Africa, the Middle East and Asia as having some of the highest prevalence rates [8]. Reports from the United Kingdom reporting sub-groups of their population, especially immigrants from Africa, the Middle East and Asia, remain at increased risk of vitamin D deficiency [8]. Worldwide public health advocates continue to recommend vitamin D supplementation for all age groups, especially pregnant or lactating mothers and children through adulthood, as nutritional supplementation remains a strategy to combat the deficiencies seen due to dietary changes, clothing protection, and shelter-restrictive habits. Cultural, ethnic, and religious standards too have played a large role in the global scale of rickets due to indoor living and in populations wearing clothing that covers the entire body [8]. Additionally in these areas, the agrarian lifestyle—which unknowingly sought nutritional answers centuries' prior—is no longer a major way of life, especially for city dwellers. As such, calcium and other nutritional deficiencies have again emerged. To complicate the matter further, the current technological revolution is changing the face of the epidemiology of bone metabolic disease. Humans are staying inside longer hours, either for work or leisure, and limiting sun exposure. Additionally, women in industrialized countries are having children later in life and, with advances in medical technology, deliveries (and affected infants) are pushing the limits of viability, further changing the epidemiology of these disorders. Within this article, we present a discussion specifically devoted to neonates and the risks, as well as development, of modern day metabolic bone disorders.

3. Neonatal metabolic bone disorders

Defined succinctly, metabolic bone disease in the neonate includes radiographic and biochemical changes together with a reduction in bone mineral content—comparable to an infant of similar size or gestational age [9]. Perhaps the greatest at-risk population for early infancy manifestations of corrupt bone mineralization is the preterm infant. The most rapid interval of *in utero* calcium accretion is during the third trimester of pregnancy, as the fetus/placenta actively extracts calcium from maternal circulation [10]. As a result, there is a severe disadvantage of calcium and phosphorus accretion in infants born prior to, or early in, the last trimester. With 80% of fetal calcium accumulation occurring during the third trimester to achieve 99% of fetal calcium stores at term gestation, the preterm infant lacks the storage capacity of their term counterparts [10]. Although only 80% of fetal phosphorus stores are accumulated within the skeleton by term gestation, phosphorus deficiency remains the primary nutritional cause of developing metabolic bone disease in the preterm infant [10]. As these infants are deprived of appropriate mineral accumulation during this phase, their skeletal structures are predisposed to metabolic bone disease [11].

With increased survival of low birth weight and preterm infants, another concern surrounding metabolic bone disease in these vulnerable infants is vitamin D deficiency. As a result of these improved survival rates, vitamin D deficiency remains a prominent problem in neonatology [12]. The etiologies of metabolic bone disease in premature infants are unique. Various prevalence rates from the 1980s cite rickets in ~50% of ELBW infants [13,14]. Thereafter, with the introduction of high mineral nutrition, prevalence reports decreased to ~30% [15]. Nevertheless, prevalence reports within the last decade continue to range as high as 40% in both ELBW and VLBW populations [9]. Various pharmacotherapeutics commonly used in neonatal intensive care units, to include diuretics, caffeine, postnatal corticosteroids, and antacids, have all been cited as risk factors for the development of metabolic bone disease [16–18]. There is a high incidence of rickets in ELBW infants with severe parenteral nutrition-associated cholestasis (PNAC), due to decreased vitamin D absorption and 25 hydroxylation, as well as renal disease, due to 1,25-hydroxylation and decreased formation of active vitamin D [17–20].

In present day, incidence rates as high as 32% of VLBW and 54% of ELBW infants having metabolic bone disease have been reported [1,21]. By far the most prevalent bone metabolic disorders of childhood are rickets and osteomalacia, due to inadequate calcium and/or phosphorus stores as well as vitamin D deficiency [22]. As compared to osteomalacia, which designates impaired bone matrix mineralization, *rickets* describes mineral deficiencies, which lead to growth plate restructuring. While the growth plates remain open in childhood, these descriptors/diagnoses may arise concurrently [23]. Due to these mineral deficiencies, architectural changes in the bone increase the size of the growth plate and metaphysis, leading to overall instability and potential for deformities (i.e. bowing, cranial bossing, craniotabes, delayed fontanelle closures, etc) [24,25]. Identifying infants, both term and preterm, who are at risk for bone mineralization disorders is paramount. Additionally, making the distinction of a short term risk of osteopenia vs a long term risk is necessary for appropriate medical management, as early intervention strategies improve long term outcomes. While appropriate *ex-utero* mineral accretion, via enteral nutrition fortification and vitamin supplementation, may correct early bone mineralization deficits in order to prevent long term complications, a number of retrospective studies have proven worthwhile in identifying distinct historical, clinical, and surgical risk factors. We present a broad overview of these risk factors.

Calcipenic rickets refers to a variety of disorders characterized by too little calcium to efficiently meet the demands of bone development. Epidemiology reports ranging from 12% up to 93% of infants in various mother-infant dyad studies from around the world confirm the most common cause of calcipenic rickets—calcium and/or vitamin D deficiency [26–29]. The most common forms of these deficiencies include dietary deficits of calcium or vitamin D, leading to insufficient intestinal absorption of calcium, and decreased vitamin D activity. This decreased gut responsiveness may be secondary to diverse metabolic causes. Early aggressive enteral feeding has the goal of decreasing the risk of rickets in previously fragile populations. Nevertheless, hypocalcemia may be the only primary symptom in the initial stage of developing rickets, while severity of disease progresses with systemic hypophosphatemia in later stages.

Unlike the normal serum phosphate levels appreciated during early hypocalcemia (vitamin D deficient rickets-stage one), the second stage of rickets bone disease typically presents with normal serum calcium [due to increased parathyroid hormone (PTH) responses] and new hypophosphatemia. Of note, low serum phosphorus with a normal/mildly elevated PTH and normal serum calcium can also be seen in X-linked hypophosphatemic rickets—a form of vitamin D resistance. Stage three of vitamin D deficiency rickets presents, most frequently, with severe clinical findings and both hypocalcemia (as PTH is no longer able to compensate) and hypophosphatemia. Although incidence reports suggest hypophosphatemia may occur in 0.2–2.2% of all inpatients, these results are difficult to assess for several reasons [30]. First, phosphorus is primarily found intracellularly, so serum concentrations may be an inaccurate predictor of true sufficiency. Second, these prevalence values are derived from adult and pediatric studies vs neonatal studies. Nevertheless, based on hypophosphatemia results in admitted pediatric intensive care patients, nutritional deficits/malnutrition in the initial three inpatient days are notorious for creating new hypophosphatemia [31]. Interestingly, Pajak *et al.* retrospectively reviewed a cohort of 24^{+0} to 32^{+6} week premature infants in the initial week of life to identify the risk of hypophosphatemia and revealed an initial threat on day of life one, as well as a secondary cohort decline between days of life four through seven [32]. In comparison with pediatric studies, this timeline of malnourishment during the first three inpatient days—for a variety of ages—appears to be significant. As such, early aggressive nutrition (both enteral and parenteral) aims to prevent these deficiencies in the short and long term.

Despite adequate nutritional recommendations and supplementation, genetic causes for metabolic bone disease cannot be overlooked. These genetic mutations and inherited disorders may present in the neonatal period, or later in childhood. Renal insufficiency, intestinal absorption, or processes involved in bone formation itself may be to blame. *CYP27B1* and *CYP2R1* mutations are known to cause vitamin D deficiency, due to their effects on the hydroxylation process. Patients with 1-alpha-hydroxlyase deficiency (*CYP27B1* mutation) are unable to convert 25(OH)D to $1,25(OH)_2D$, and thus require $1,25(OH)_2D$ replacement therapy [33]. Similarly, in 25-hydroxylase deficiency (*CYP2R1* mutation), the gene encoding the primary enzyme responsible for 25-hydroxylation of vitamin D is defective, warranting high-dose vitamin D and calcium supplementation for treatment purposes. Lastly, an extremely rare autosomal recessive disorder (~100 historically reported cases) known as hereditary resistance to vitamin D is caused by mutations in the VDR leading to end-organ resistance [34].

Although genetic and inheritable disorders are rare etiologies of rickets, iatrogenic and post-surgical complications are not uncommon in the pediatric population—nor are these obstacles selective to the neonatal population. A variety of neonatal surgical complications, affecting both preterm and term infants, culminate in absorbency-related issues of the gastrointestinal (GI) tract. Necrotizing enterocolitis, spontaneous bowel perforations, and various intestinal atresias compile a large number of short-gut syndromes in preterm infants, which predispose to intestinal malabsorption. What is most important to review is the area of the gut that is involved in calcium absorption, as various diagnoses may or may not involve these areas. Primarily two mechanisms exist for the absorption of calcium, which includes a paracellular passive approach through the length of the small intestine, and a transcellular active transport method. The later of these absorptive mechanisms occurs primarily in the duodenum and proximal jejunum. Interesting enough, Bronner describes a downregulatory action of the transcellular active transport receptors occurring when enteral calcium intake is high. Likewise, an upregulation of these receptors is seen in the duodenum and jejunum when enteral calcium intake is low [35]. As such, the limits of active absorption are compromised with various post-surgical bowel resections or atresias involving the duodenum and jejunum. In cases of necrotizing enterocolitis with subsequent bowel resection, or alternate post-surgical short-gut complications, the passive process of calcium absorption is compromised and severity of resultant hypocalcemia may vary. By appreciating the small intestine as the principal region of absorption, clinicians can suspect alternative etiologies when hypocalcemia presents in children with distal (i.e. colonic) surgical and non-surgical diagnoses.

It is not surprising, however, that the post-surgical management of many of these GI disorders involves long term parenteral nutrition, which predisposes these infants to cholestasis. Additionally, alternative liver disorders, such as biliary atresia, Alagille syndrome, and various metabolic disorders, may act in a similar manner. Neonatal cholestasis involves a rapid sequence evaluation to identify infants who need an immediate or early surgical evaluation vs those requiring supportive care. For those infants who require long-term parenteral nutrition, cholestasis is a product of decreased or delayed enteral nutrition. Specifically, infants born prematurely and in those who have short bowel syndrome or intestinal failure, PNAC commonly develops in those receiving parenteral nutrition (PN) for more than two to four weeks. In these infants, nutritional therapy is imperative, as growth failure is commonly seen due to impaired absorption of fats, proteins and carbohydrates as well as a reduced delivery of bile salts, which subsequently decreases fat-soluble vitamin absorption (i.e. vitamin D, E and K) [36]. Interestingly, patients who have chronic cholestasis but have corrected 25(OH)D concentrations, continue to have metabolic bone disease, which suggests alternate contributions to the disease process—not solely vitamin D deficiency due to malabsorption [37].

Infants, such as those aforementioned with cholestasis, who demonstrate altered postnatal linear growth have definitive risks for the development of osteopenia. Likewise, the incredible biotransformation of vitamin D prenatally provides further evidence of the placental role in the development of bone mineral content. In this regard, small-for-gestational-age infants and those with intrauterine growth restriction, may display poor bone mineral content at birth. In preterm infants, cortical growth itself may be diminished for various reasons, such as limited *in utero* time to build strength via mechanical forces (i.e. kicking) against the uterine wall. Each of these potential fetal setbacks have the opportunity for repair postnatally, but may predispose these infants to further long term risks of osteopenia. Alterations in long bone density over the initial six months of life may reflect differences between pre- and postnatal hormonal profiles. In infants who display extrauterine growth restriction, signs of osteopenia may not manifest until periods of more rapid bone growth. In these initial six months of life, due to the increased postnatal growth of the bone marrow cavity as compared to the cross-sectional area of the bony cortex, the long bone density may decrease up to 30% [38]. Identifying these radiologic features, however, may prove to be more difficult in the earlier stages of bone metabolism disorders. The current gold standards for screening metabolic bone disorders and the future possibilities are further discussed.

Although the majority of emphasis in nutritional optimization in preterm infants, as it relates to bone health, has been centered on vitamin D, calcium, and phosphate maximization, an evolving niche of parenteral nutrition-related studies investigating alternate nutrients that contribute to metabolic bone disease is rising. Despite previous beliefs suggesting single fatty acids were independent contributors to health and disease, the impact of polyunsaturated fatty acids in—specifically—parenteral nutrition is now known to be dependent on the total fatty acid profile. Studies utilizing new intravenous fat emulsions in preterm infants have shown attenuation of diagnoses in this population which are established risk factors for the development of metabolic bone disease [39]. In one recent study evaluating the effects of two types of fat emulsions [soybean oil vs soybean oil, medium-chain triglyceride oil, olive oil, and fish oil (SMOF)] in VLBW infants, the results yielded evidence to support the use of SMOF, as it significantly lowered serum alkaline phosphatase concentrations by day 45 of life and at hospital discharge, compared to those who received solely soybean oil [40]. Additionally, the infants who received SMOF fat emulsions during their course of parenteral nutrition were found to have significantly higher serum phosphate levels at hospital discharge [40]. As these two findings would argue a potential reduction in the relative risk for the development of metabolic bone disease with the use of SMOF fat emulsions, larger additional studies are warranted to evaluate long-term and specific outcomes related to the source of parenteral nutrition fat emulsion oil sources. Radiologic corroboration of these specific parenteral nutrition-related clinical findings is also limited in the current literature.

4. Nutritional correlations of metabolic bone disorders in prematurity

Although the most common forms of rickets may occur with adequate levels of vitamin D in the setting of low calcium intake, the combination of adequate vitamin D intake with hypocalcemia is rare—given vitamin D's ability to increase intestinal absorption of calcium [41,42]. As vitamin D turnover is accelerated in the setting of low serum calcium, vitamin D requirements may be higher in calcium-deficiency [43,44]. In 2008, the AAP reviewed their policy and enacted strict guidelines/recommendations for vitamin D supplementation in neonatal and pediatric patients. These recommendations were echoed in 2016 with the *Global Consensus Recommendations on Prevention and Management of Nutritional* Rickets [45]. Current AAP recommendations suggest 400 international units (IU) of daily supplementation may be necessary to achieve normal vitamin D status in those infants who may have an increased risk of vitamin D deficiency. Interestingly, the European guideline on daily vitamin D supplementation recommends 800–1000 IU/day for preterm infants [12]. However, no existing recommendations by the AAP or Institute of Medicine (IOM) specifically comment on preterm infants.

With various worldwide arguments on the need, dose, and duration of vitamin D supplementation in preterm infants—especially ELBW and VLBW populations—several studies have revealed safety and efficacy in the treatment of these specialized populations with 800 IU/day, in accordance with the European guidelines [46, 47]. Additionally, a Cochrane review is currently investigating the effectiveness of a daily recommended dose of vitamin D in preterm and low birth weight infants [48].

As previously reviewed, stores of calcium, phosphorus, and vitamin D at birth are largely dependent on gestational age, maternal deficiencies, and placental sufficiency. Since requirements postnatally depend on these stores, alimentary demands differ between preterm patients and their source of nutrition. Table 1 summarizes the form of vitamin D found in various neonatal nutritional sources, assuming approximately 150 mL/kg/day in total fluids for parenteral nutrition and 160 mL/kg/day of feeding volume—once the infant is receiving full enteral feeds [49–51]. To achieve currently recommended vitamin D intake requires cautious consideration of both enteral feeding capabilities as well as additional daily supplementation.

Table 1
Nutritional correlations of vitamin D intake in premature infants.

Vitamin D form	Form of Nutrition	Vitamin D provided by daily dose*
D_2	Total parenteral nutrition	160 IU/kg/day
D_3	Human breast milk	3–4 IU/kg/day
D_3	Human breast milk + human milk fortifier	323–364 IU/kg/day
D_3	Preterm formula	192–216 IU/kg/day
D_3	Nutrient-enriched post-discharge formula	109–122 IU/kg/day
D_3	Standard infant formula	77–86 IU/kg/day

*International units (IU) based on 150 mL/kg/day of total parenteral fluid intake or 160–180 mL/kg/day of oral/enteral feeds.

Unfortunately, practice differences exist in discharge planning among many tertiary care centers in the United States. As a result of this variation in discharge feeding plans, risks of bone mineralization disorders persist in exclusively breastfed preterm infants whose diets remain unfortified post-hospital discharge. Despite improved developmental outcomes in term breastfed infants, human milk alone may have insufficient nutrient content to support optimal growth in the preterm infant. While the advantages of human milk are well established in the protection against necrotizing enterocolitis and sepsis, opinion variance exists on short vs long-term neurodevelopmental outcomes in post-discharge fortification of human milk in preterm infants. The proposed advantages of higher protein and nutrient content of fortified post-discharge breast milk aim to improve these outcomes [52]. Nevertheless, a spectrum of extrauterine growth failure remains in many preterm populations. For those receiving donor breast milk or mother's-own breast milk as compared to formula, great care must be taken to ensure appropriate growth trajectory due to the notorious poor growth seen in many VLBWs receiving exclusive human milk diets. Nevertheless, O'Connor *et al.* found better weight, length, head circumference, and bone mineral density in infants < 1250 g (birth weight) with fortification of breast milk post-discharge; however, neurodevelopmental outcome at 12–18 months was not different between human milk fortifiers and preterm formula [53]. Research on individualization (as compared to the previous method of standardization) of fortified human milk feeds post-discharge will yield the next stages of optimizing infant nutrition in premature neonates.

5. Long term outcomes

While early nutritional goals in the preterm neonate aim toward structuring stronger and more normalized bone matrices, abnormal mineralization creates a fear of future fractures in these infants. Birth injury-related fractures are most common in term infants with difficult deliveries and include clavicular, humeral, and femur fractures [54]. These fractures, most commonly, represent normal bone architecture with undue stress. However, more recent evidence validates previous findings that outside of early infancy, prematurity alone may not confer an increased risk of fracture [55]. Major risk factors concerning for neonatal osteopenia, and posited in the increased risk of fractures during the first year of life, include diuretics administration, dexamethasone administration, methylxanthines, histamine-2 receptor blockers (H_2 blockers), proton pump inhibitors (PPIs), as well as additional pathologic conditions seen in many preterm infants—such as bronchopulmonary dysplasia and necrotizing enterocolitis [38].

Although many infants at risk for metabolic disease present during their critical care inpatient course, there remains a variety of genetic conditions, typically presenting in the neonatal period but may not present until childhood, which notoriously cause clinical bone fragility—namely, fractures. Prenatal diagnosis is often difficult for various skeletal dysplasias, but many of the severe signs, such as contractures and decreased fetal movement, are present *in utero* and lead to an early diagnosis and prognosis postnatally. Unfortunately, fractures occurring in children of any age—especially neonates—deserves additional attention for possible maternal historical clues, which may explain inappropriate mineralization in the setting of suitable nutrition. Pregnancy history often details early signs for increased risks of metabolic bone disease in infants. Maternal vitamin D deficiency, gestational diabetes, and certain maternal medications (primarily those which interfere with calcium and phosphorus absorption, such as antacids) have each been shown to cause a temporary risk to the infant [56, 57]. Similarly, decreased placental loading/transfer of calcium, phosphorus, and vitamin D due to various vascular disorders of pregnancy result in intrauterine growth restriction and an increased likelihood of baseline nutritional deficiencies in these infants [58].

When repeat or recurrent fractures are noted, the differential diagnosis may change and alternative genetic or endocrinologic disorders may warrant further evaluation. In fracture cases where child abuse may be suspected or misinterpreted, a thorough parental history, serum biomarkers, and potentially DNA sequence analysis may be helpful in identifying etiologies for fragility fractures in infants. Although non-accidental trauma should always be considered as a potential cause for multiple fractures in various stages of healing, inherent bone disorders may be to blame and require an extensive evaluation [59–61]. As varying suspicious exam findings, such as unexplained bruising, soft tissue injuries, and dislocations may be common in physical abuse, these same findings can also naturally occur in varying degrees in some types of Ehlers-Danlos syndrome (EDS). Likewise, the same form of EDS may have a spectrum of presentations between affected individuals. In conditions such as osteogenesis imperfecta (OI), some rare types of EDS, and polyostotic fibrous dysplasia (characteristic of McCune-Albright syndrome), fractures may occur spontaneously or with exceedingly minimal trauma. However, The Ehlers-Danlos Society stated (as of 2018) there was no evidence to support fractures occurring in this manner in children with the hypermobile variant of EDS. This specific variant has been cited as the reason for fractures in numerous trials and child abuse cases over the previous decade [60], blurring the lines of genetic screening. This variance in opinion presents itself in the wake of newer technology demonstrating a spectrum of disorder presentations, especially in the neonatal and pediatric populations.

6. Future research endeavors

As clinicians hone the markers that predispose preterm and term neonates to the development of metabolic bone disease, early biochemical detection will allow for earlier assessment of bone health. There is mounting evidence that supports the use of early enteral nutrition, with appropriately matched parenteral nutrition until full volume fortified feeds can be established, in critically ill infants (especially ELBWs and VLBWs). Further, the importance of vitamin supplementation that include vitamin D, and the need for continued fortification of both breast milk and formulas to deliver adequate calcium and phosphorus beyond the NICU period is underscored by historical and recent studies. With these interventions, there is an expectation to decrease the future risk of bone metabolic disorders in premature infants. Likewise, strategic and *limited* use of medications with historical extensive use in the NICU, such as diuretics, postnatal corticosteroids, and antacids, should aim to decrease this metabolic bone disease risk as well. Optimization of standardized feeding protocols and parental nutrition protocols with adherence of these protocols provides the additional benefit of identifying the abnormal case or those infants with increased rickets risks factors. It additionally provides a baseline for identification of infants who may have an underlying genetic or endocrinologic disorder which predisposes to bone demineralization.

6.1. *Practice points*

- The evolution and migration of humans across a wide array of latitudes has led to significant epidemiological changes in bone metabolic disease.
- Cultural, traditional, ethnic, and religious standards as well as the current technological era play a role in the prevalence of modern-day rickets.
- The limits of gestational viability and medical advances have altered the nature of neonatal metabolic bone disease in recent decades.
- Optimization of nutritional standards, vitamin supplementation, and new diagnostic technology will play a future role in neonatal bone metabolism.

6.2. *Research directions*

- The role of standardized maternal vitamin D supplementation in disease development across at-risk populations.
- Longitudinal studies on premature infant bone development through adulthood, given current recommendations on vitamin supplementation and early lipid administration.

Declaration of competing interest

The authors have no disclosures or conflicts to declare.

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Seminars in Fetal and Neonatal Medicine 25 (2020) 101076

Contents lists available at ScienceDirect

Seminars in Fetal and Neonatal Medicine

journal homepage: www.elsevier.com/locate/siny

Mineral and bone physiology in the foetus, preterm and full-term neonates

Amanpreet Sethi[a], Mayank Priyadarshi[b], Ramesh Agarwal[b,*]

[a] Department of Pediatrics, Guru Gobind Singh Medical College and Hospital, Faridkot, Punjab, India
[b] Division of Neonatology, Newborn Health Knowledge Center (NHKC), Department of Pediatrics, All India Institute of Medical Sciences, New Delhi, India

ARTICLE INFO

Keywords:
Mineral physiology
Bone physiology
Foetal
Neonatal
Calcium
Phosphorus
Magnesium

ABSTRACT

Mother is the major source of minerals in foetal life with placenta actively transporting against a concentration and electrochemical gradient. The foetal serum mineral concentration is thereby higher as compared to maternal values, which possibly help in its rapid accretion in developing bones and for counteracting postnatal fall in calcium levels at birth. Parathyroid hormone related peptide (PTHrP) and parathyroid hormone (PTH) play a major role in mineral physiology during foetal life with hormones like calcitriol, calcitonin, FGF-23 and sex steroids having minimal role. PTHrP and PTH also play a major role in endochondral bone formation and mineralization of skeleton. At the birth, as the cord is clamped, there is loss of active transport of minerals through placenta and the neonate has to rely on enteral intake of minerals to meet the demands of growing bones and metabolisms. The calcium levels fall after birth, reaching a nadir at 24–48 h and gradually rise to adult values over several days, probably resulting from a fall in PTHrP levels and hyporesponsiveness of parathyroid glands. As PTH and calcitriol levels increase postnatally, there is a rise in calcium levels with maturation in functioning of kidneys and intestines. However, there may be significant delay in intestinal maturation in preterm infants along with an increased demand for mineral accretion, which predispose them to osteopenia of prematurity.

1. Introduction

Calcium (Ca), phosphorus (Pi) and magnesium (Mg) are the major minerals involved in mineralization of bones in foetus, neonate and adult but their homeostasis and regulation in the foetus and early neonatal period is considerably diverse from children and adults [1]. Hormones like calcitonin; sex steroids like oestrogen and testosterone; vitamins like vitamin D and fibroblast growth factor-23 (FGF-23) which play an important role in adults and children are not at all required for in-utero transfer of minerals from placenta to foetus and regulation of bone mineralization [2]. Foetal bone mineralization is primarily regulated by parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP). After birth, as mineral transfer from placenta is interrupted, there is a transition from foetal to neonatal regulation characterised by a fall of serum Ca and a rise of Pi leading to increase in serum PTH and vitamin D levels. The major source of Ca, Pi and Mg changes from placenta to intestines while kidneys adopt the role of maintaining a fine balance of these minerals in serum and bone [3] (see Fig. 4)

Majority of the in-utero mineral transfer occurs in the last trimester of pregnancy characterized by high cord blood Ca levels as compared to maternal serum [4]. Serum Ca levels in foetus are around 10–11 mg/dl, which is 1–2 mg/dl higher than the maternal level, due to active transfer of Ca across placenta against a concentration gradient [5]. The human foetus accretes Ca at a rate of 120–140 mg/kg/day and Pi at a rate of 75–90 mg/kg/day in the last trimester [6]. This high mineral concentration at the end of gestation is essential for bone mineralization. Most of our understanding of mineral and bone physiology in foetus and neonate is based on animal studies using diverse animal models and employing different approaches viz. surgical, pharmaceutical and genetic. The recent use of novel genetic approaches like total and partial gene deletion models have allowed us to understand the role of PTHrP, PTH, vitamin D and its receptor, calcitonin, and FGF23 in bone and mineral physiology in the foetus and neonate [1]. Human studies have been limited on this aspect except the role of vitamin D which has been extensively studied.

In this review, we will discuss foetal bone and mineral homeostasis along with Ca, Pi and Mg homeostasis in term and preterm neonates.

Abbreviations: PTHrP, Parathyroid hormone related peptide; PTH, Parathyroid hormone; FGF-23, Fibroblast growth factor-23; CaSR, Calcium sensing receptor; Ca, Calcium; Mg, Magnesium; Pi, Phosphorus; TRPV6, Transient receptor potential cation channel, subfamily V, member 6; NCX1, Sodium-calcium exchanger

[*] Corresponding author. Dept. of Pediatrics, All India Institute of Medical Sciences, New Delhi, 110029, India.
E-mail address: ra.aiims@gmail.com (R. Agarwal).

https://doi.org/10.1016/j.siny.2019.101076

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Abbreviations

PTHrP	Parathyroid hormone related peptide;
PTH	Parathyroid hormone
FGF-23	Fibroblast growth factor-23
CaSR	Calcium sensing receptor
Ca	Calcium
Mg	Magnesium
Pi	Phosphorus
TRPV6	Transient receptor potential cation channel, subfamily V, member 6
NCX1	Sodium-calcium exchanger

2. Foetal bone and mineral homeostasis

2.1. Serum mineral concentration in the foetus

Mineral concentration in foetal serum is universally higher as compared to maternal serum across all mammalian species to meet rising mineralization demand of the developing skeleton.

a) **Serum calcium**: Serum Ca in foetus is maintained at 0.3–0.5 mM/L higher than the maternal level [4,7,8]. The two theories which have been put forward to explain this phenomenon are either presence of novel Ca sensing receptor (CaSR) which sets Ca target at a higher level in foetus or active transfer of Ca from mother to foetus [1]. Considering the latter to be true, there should have been a gradient between maternal and foetal serum concentration and low serum Ca in mother would lead to low levels in foetus. Though, on the contrary numerous animal studies have revealed that foetal serum Ca level remains constant and is not affected by maternal hypocalcaemia caused by either decreased Ca intake, vitamin D deficiency or low PTH [9–11]. Hence, the former theory seems to be more plausible.

b) **Serum phosphorus:** Pi in foetus is maintained around 0.5 mM/L higher as compared to maternal levels [12,13]. Pi is important for endochondral bone development as it plays an important role in apoptosis of chondrocytes and formation of osteoid [14]. Limited animal studies have shown that serum Pi also remains constant even in the setting of hypophosphatemia in pregnant animals [15].

c) **Serum magnesium:** Mg is maintained at just 0.05 mM/L higher as compared to maternal levels. [4,16] Mg is crucial for synthesis of biomacromolecules (DNA, RNA and proteins), bone matrix development, energy production as well as nerve and muscle function [17]. Serum Mg concentration is also set independent of maternal serum values but a recent meta-analysis correlating cord blood Mg levels with maternal serum values in term and preterm human neonates has shown that cord blood Mg levels are affected by maternal Mg supplementation [18].

2.2. Regulation of serum mineral concentration in the foetus

Serum mineral concentration is primarily regulated by PTH and PTHrP in the foetus. Foetal circulation is characterized by high levels of PTHrP and calcitonin and low levels of PTH and vitamin D [3].

a) **PTHrP:** Early human and animal studies showed high PTH like activity with very low levels of immunoreactive PTH. It was later found that this high PTH like activity was due to PTHrP characterised by availability of specific radio-immunoassays. The mid-molecular form of PTHrP is the most active form which facilitates Ca transfer across the placenta [3]. The major source of PTHrP appears to be the placenta as the loss of parathyroid gland in foetus does not lead to any decrease in PTHrP levels [19]. PTHrP is essential for regulation of foetal serum Ca levels as loss of PTHrP activity in foetus leads to fall of Ca levels equivalent to maternal blood levels with 3-fold rise in PTH levels because of activation of CaSR [20,21].

b) **PTH:** Foetal parathyroid gland starts secreting PTH as early as the 10th week of gestation [22]. The role of placenta in secreting PTH is still not clear. As discussed earlier, multiple animal and human studies have revealed low PTH levels secondary to high serum Ca in the foetus as compared to maternal values [1]. Though maternal PTH does not cross placenta, perturbations of maternal serum Ca levels can affect foetal PTH status. Maternal hypercalcemia leads to increased Ca levels in foetal circulation resulting in foetal PTH suppression which can later lead to increased chances of neonatal hypocalcaemia and its associated symptoms [23]. These findings have been observed in babies born to mothers who had hypercalcemia during pregnancy due to primary hyperparathyroidism, inactivating mutation of CaSR [24] or hypercalcemia of malignancy [25–27]. On the contrary, maternal hypocalcemia due to any cause (hypoparathyroidism or pseudohypoparathyroidism) results in foetal parathyroid gland hyperplasia with increased PTH secretion resulting in increased resorption, demineralization and fractures of the foetal skeleton [28].

c) **Vitamin D**: The major source of the active form of vitamin D (calcitriol) is the foetal kidney as suggested by higher calcitriol levels in umbilical arteries compared to umbilical vein [29]. Vitamin D level in foetal blood is usually 50% of the maternal value. The major circulating form of vitamin D in foetal blood is 25-hydroxyvitamin D as it easily crosses placenta [30]. Calcitriol synthesis in foetus is probably decreased by high serum Ca, Pi and low PTH concentrations. It has also been shown that human trophoblasts and maternal decidua express 1α-hydroxylase and convert 25-hydroxyvitamin D to calcitriol but there is preponderance of 24-hydroxylase activity leading to 30 to 40-fold higher concentration of 24,25-dihydroxyvitamin D (inactive form) instead of calcitriol [31].

d) **Calcitonin:** Calcitonin is produced by thyroid C-cells around the 12th week of gestation [32]. The major source appears to be foetal thyroid gland, though placental trophoblasts also secrete calcitonin and may, therefore, contribute to total amount in foetal circulation. Serum calcitonin is almost twice the maternal level in foetus, probably due to high Ca concentration in the foetal blood [29,33].

e) **Fibroblast growth factor-23** (FGF-23): The major source of FGF-23 are osteocytes and osteoblasts. It is a phosphorus-regulating hormone and helps to maintain adequate concentration of Pi at level of bone surface thus enabling appropriate mineral accretion [34]. It decreases serum Pi levels by downregulating sodium-phosphate cotransporters 2a and 2c (NaPi2a and NaPi2c) in proximal renal tubules and 2b (NaPi2b) in intestines, thus increasing renal excretion and decreasing intestinal absorption. This action is mediated via decreased calcitriol synthesis as FGF-23 inhibits 1 α-hydroxylase activity. Human studies have shown that cord blood contains intact FGF-23 levels at 1/3rd to 1/6th of maternal values and 6 times the levels of FGF23 coreceptor Klotho. Thus, FGF-23 actions in foetal period are limited thus allowing higher concentration of Pi in foetal serum [35].

2.3. Role of foetal kidneys

Their role in regulation of mineral and bone homeostasis is limited as majority of mineral transfer occurs through the placenta. Most of the minerals, excreted as urine in the amniotic fluid, is again reabsorbed by the foetal intestines and thus recycled [1]. This does not mean that renal tubules are not responsive to calcio-trophic or phospho-trophic hormones as animal studies have revealed decreased Ca excretion in response to PTH infusions.

2.4. Role of foetal intestines

In adults, the major route of mineral entry is through intestines and the major regulatory hormone is activated form of vitamin D (calcitriol). Several animal studies involving rats have shown the absence of vitamin D receptors in foetal enterocytes. Thus Ca and Pi absorption is largely passive and non-saturable [36,37]. Similarly, studies in human preterm infants whose intestines behave like the foetal intestines show similar findings of passive dose dependant Ca and Pi absorption with no response to therapeutic doses of calcitriol [38,39].

2.5. Role of placenta

Human placenta is disc-shaped with finger like projections organ invading uterine wall. It is also haemomonochorial which means that it lies in a pool of maternal blood with single layer of trophoblast separating foetal and maternal blood [40]. Placenta has a major role to play in transferring minerals to foetus with almost 80% of the transfer occurring in third trimester [1]. Various mechanisms for mineral transfer are described below:

a) **Calcium transfer:** The majority of Ca transfer across placenta is by an active process; active transport peaks at the end of gestation as shown by in-vitro perfusion studies in rodent and human placentas [41,42]. Various channels that are involved in active transport are Ca channels [such as transient receptor potential cation channel, subfamily V, member 6 (TRPV6)] present on basement membranes facing maternal circulation and allowing Ca entry into trophoblastic cells. Within these cells, Ca binding protein, Calbindin-D9k, transports Ca to the opposite membrane which is actively pumped by Ca^{+2}-ATPase at foetal-facing basement membranes into foetal circulation [43]. In the third trimester, there is an increased expression of calciotropic genes resulting in augmented formation of Ca transport channels, enzymes and Ca binding proteins.
b) **Phosphorus transfer**: Mechanisms of Pi transfer across the placenta are less well studied as compared to Ca [1]. Radiolabelled P^{32} transport studies utilizing rat placenta have revealed increasing Pi transfer across placenta till term gestation [44]. As discussed earlier, placenta also express phosphate regulating genes, including sodium-phosphate channels NaPi2a, NaPi2b and NaPi2c; FGF-23 coreceptor Klotho, and all four FGF receptors [35].
c) **Magnesium transfer:** Though not extensively studied, an invitro perfusion study of isolated human placental lobules has suggested active transport as the major mechanism [45].

2.6. Foetal bone development and mineralization

Bone development and mineralization is an interplay of various genes and regulatory factors like HOX genes, WNTs, Hedgehogs, bone morphogenetic proteins, fibroblast growth factors, Notch/Delta etc which has been studied in detail in a mouse model [46].

a) **Patterns of ossification**: Bone formation exhibits two patterns of ossification: endochondral and intramembranous. Intramembranous ossification is seen in all skull bones except mandible [1]. Here, mesenchymal cells directly differentiate into osteoblasts. The majority of bones in human skeleton is formed by endochondral ossification. In this process, mesenchymal cells first differentiate into chondrocytes and then, through multiple stages, endochondral bone is formed (Fig. 1) [47].

The cartilaginous skeleton of human foetus and primary ossification centres in vertebrae and long bones are completed in the first trimester. But, the majority of mineral accretion occurs in the third trimester [48].

a) **Regulation of bone ossification**:

PTHrP and PTH hormones are the major regulators of bone ossification. PTHrP, secreted by perichondral cells and proliferating chondrocytes, postpones the terminal differentiation and hypertrophy of chondrocytes [49,50]. In absence of PTHrP, there is a hypertrophy of chondrocytes leading to an early apoptosis resulting in chondrodysplasia with short long bones [18–20]. PTH mainly regulates accretion of minerals in foetal skeleton, but its role is not clear in endochondral bone formation [51].

3. Neonatal bone and mineral homeostasis

3.1. Calcium

1. **Body Distribution:**

Ca **is** the most abundant mineral in body required for bone mineralization and normal cellular functions. The total body content increases from new-born age to adult age from approximately 30 gm at term gestation to around 1–2 kg in adults. In newborn infant, 99% of the total Ca content is in bones with only 1% in extracellular fluid (ECF), intracellular fluid (ICF) and soft tissues**.** Plasma Ca exists in 3 forms: ionized Ca (iCa^{+2}) (50%); bound to proteins, predominantly albumin (40%); complexed with citrate, phosphate and bicarbonate (10%) [52].

2. **Hormonal Regulation of serum Ca concentration:**

Calcitriol, PTH and calcitonin are the main hormones which regulate serum Ca concentrations in neonate. After birth, plasma Ca concentration declines during first 24–48 h of life due to loss of placental supply along with parathyroid gland hypo-responsiveness [53]. Ca concentration subsequently increases from newborn to adult age. The role of the above-mentioned hormones is summarized in Table 1.

3. **Role of gastrointestinal (GI) tract:**

GI tract is the main site for absorption of Ca after birth. Major insight on the mechanisms of Ca absorption in GI tract comes from studies on rat intestines [54,55]. GI Ca absorption occurs by two main mechanisms: transcellular and paracellular (Table 2, Fig. 2). Many factors

Table 1
Role of calciotropic and phosphotropic hormones.

	PTH	Calcitriol	Calcitonin
Homeostasis affected	Ca and P	Ca and P	Ca
Stimulus for secretion	↓ Ca, ↑ P levels	↓ Ca, ↓ P levels	↑ Ca level
Action on bone	↑ Resorption	↑ Resorption	↓ Resorption
Action on GIT	–	↑ Ca and P absorption	–
Action on kidneys	↑ conversion to calcitriol, ↓ Ca and ↑ P excretion	↓ Ca, P excretion	↑ Ca, P excretion
Net effect	↑ Ca, ↓ P	↑ Ca, ↑ P	↓ Ca

Table 2
Mechanisms of calcium absorption in GI tract [56].

	Transcellular	Paracellular
Energy dependant	Active, three step process	Passive through tight junctions
Predominant site	Duodenum and upper part of jejunum	Whole length of intestine
Regulation of absorption	Vitamin D dependant	Concentration and electrochemical gradient dependant
Enhanced absorption	During low calcium intakes	During high calcium intakes, also enhanced by bile salts and lactose in the terminal ileum

play a role in GI absorption of Ca in term and preterm neonates as depicted in Fig. 3.

4. **Role of kidneys:**

Kidney regulates Ca excretion and reabsorption in response to serum concentrations with help of Ca sensing receptors (CaSR) [52]. Studies have shown around 98% of the filtered Ca is reabsorbed by similar transcellular and paracellular mechanisms described above in GI tract (Table 3). Most Ca is reabsorbed passively by paracellular mechanism −70% in proximal tubules and 20% in thick ascending loop (TAL) of Henle. The remaining 10–15% is reabsorbed by an active transcellular route in distal segments of nephrons [57].

3.2. Phosphorus (Pi)

1. **Body distribution**:

Like Ca, Pi is also one of the main constituents of human skeletal system (85% of total body Pi). Approximately 15% of the remaining body Pi is in form of inorganic phosphate and phosphate ester in ECF and soft tissues respectively. Serum Pi in ECF exists in three forms: ionized Pi (55%); protein-bound Pi (11%); and Pi complexed to sodium, Ca, and Mg (34%). It is involved in all energy dependant cellular activities of body. In neonates at term, the total amount of Pi in body is 16 g with 80% of total Pi accreted in the last trimester at a rate of approximately 75 mg/kg/day [52].

2. **Regulation of serum concentration:**

Kidneys in conjunction with PTH are primarily responsible for regulation of serum Pi concentration by means of decreasing its tubular reabsorption in response to decreased serum Ca concentration [53]. Other hormones like growth hormone, insulin and insulin-like growth factor also regulate Pi concentration by increasing tubular Pi reabsorption. Regulation of dietary Pi absorption from GI tract is through co-transportation of sodium and Pi (Na-Pi 2b) at luminal brush-border surface. Role of vitamin D in regulating Pi absorption by stimulating Na-Pi 2b transporter is evident by the fact that there is increase in calcitriol concentration during selective low Pi intake [58,59]. Though, in preterm and term neonatal studies it has been shown that absorption of Pi from GI tract is largely vitamin D independent, unlike Ca absorption [60,61].

3. **Role of GI tract:**

Studies on Pi absorption from GI tract are limited as compared to studies on Ca. Animal studies have suggested that Pi absorption is dependent on dietary Pi intake and ensues across whole small intestine [52]. The fractional absorption of Pi is high, around 80–90%, in term and preterm neonates irrespective of dietary Pi intake [38]. In a study done in rats using radiolabelled P^{32} meal, duodenum and upper jejunum showed maximum absorption rate. The terminal ileum, because of its long transit time contributed one-third of total Pi absorption [62,63]. Similar to Ca absorption, mechanisms of transcellular (active, energy-dependent transport) and paracellular (passive, diffusional) absorption exist for Pi absorption also. Transcellular transport of Pi is dependent upon sodium-dependent co-transporter isoform Na-Pi 2b in apical membrane of enterocytes, which is also considered as the rate limiting step [63]. The factors affecting GI absorption of Pi are shown in Fig. 5.

4. **Role of Kidneys**

As discussed earlier, kidneys play a major role in serum Pi regulation. The majority of plasma Pi (90%) which is not protein bound is freely filtered in glomerulus out of which 75–85% is reabsorbed in renal tubules with remainder acting as a titratable acid in urine [52]. Tubular reabsorption of Pi is a saturable process and is measured by a coefficient known as transport maximum (TmPi) standardized according to glomerular filtration rate (GFR) for comparison. As Pi is an important constituent required for growth, TmPi/GFR is set at a higher threshold in newborn period leading to higher phosphate reabsorption. Maximum reabsorption occurs at the level of proximal convoluted tubule [64]. Pi re-absorption varies between cortical and medullary nephrons, with latter having more reabsorption capacity, probably due to early development of juxta-medullary nephrons during nephrogenesis [65]. The majority of Pi transport in renal tubular cell is by transcellular mechanism using sodium-phosphate transporters (Na-Pi) to gain entry into cell. The most important subtypes are: Na-Pi 2a and Na-Pi 2c [66,67]. Passage of Pi is mediated by an electrochemical gradient for sodium by sodium-potassium (Na^+-K^+) ATPase pump in basolateral membrane of renal tubular cell. Genetic studies involving hereditary hypophosphatemic rickets patients have revealed single nucleotide polymorphism involving Na-Pi 2c transporter gene (SLC34A3) on the Chromosome 9q34 as one of the causative mechanisms. This shows that Na-Pi 2c might be the predominant co-transporter of Pi in humans [68]. The factors affecting Pi reabsorption in renal tubular cell are shown in Fig. 6.

3.3. Magnesium (Mg)

1. **Body distribution:** Mg is an important constituent of bone, while in intracellular compartment, it acts as a co-factor for many enzymatic **reactions** along with regulation of neuromuscular excitability [52]. Total body Mg content in a term neonate is around 0.8 g with

Table 3
Mechanisms of calcium reabsorption in renal nephron [57].

	Transcellular	Paracellular
Energy dependant	Active, three step process (10–15% of total reabsorption)	Passive through inter-cellular spaces (85–90% of total reabsorption)
Predominant site	Distal segments of the nephron	Proximal tubules (70%); thick ascending loop of Henle (15–20%)
Regulation of absorption	By interplay of PTH, calcitriol, calcitonin and CaSR	Electrochemical gradient

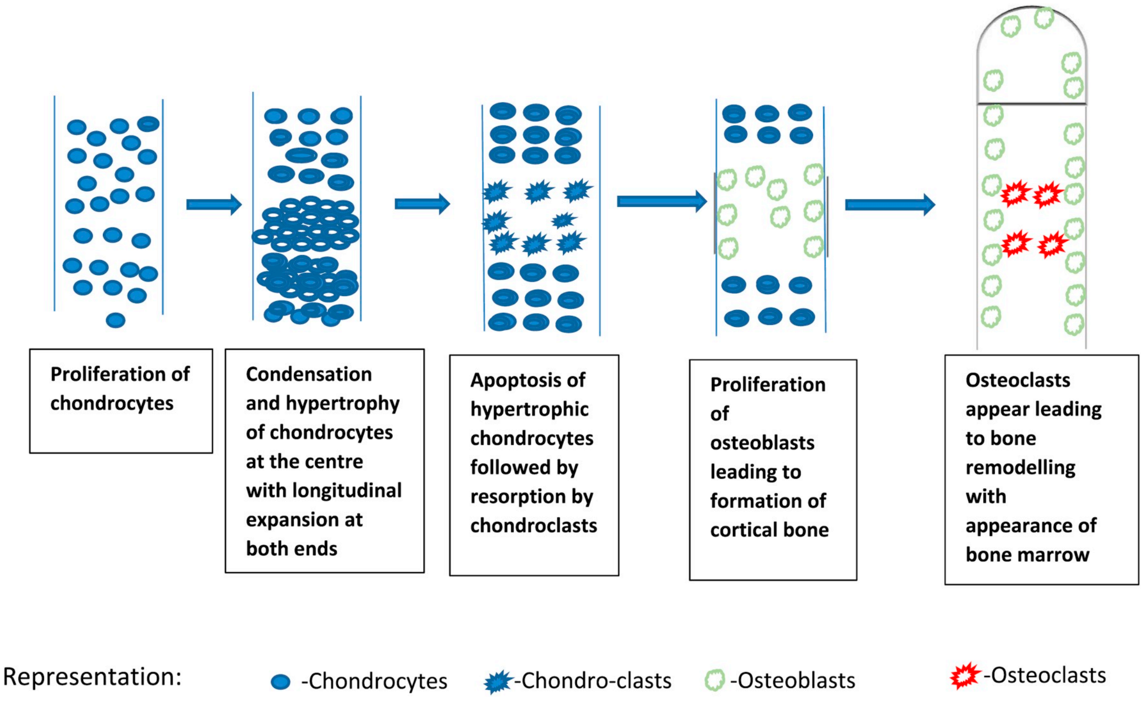

Proliferation of chondrocytes
Condensation and hypertrophy of chondrocytes at the centre with longitudinal expansion at both ends
Apoptosis of hypertrophic chondrocytes followed by resorption by chondroclasts
Proliferation of osteoblasts leading to formation of cortical bone
Osteoclasts appear leading to bone remodelling with appearance of bone marrow
Representation:
-Chondrocytes
-Chondro-clasts
-Osteoblasts
-Osteoclasts

Fig. 1. Longitudinal view of endochondral bone formation.

majority accreted in the last 3 months of pregnancy. Total-body Mg is divided into 3 major compartments of body: 65% in bones; 34% in intracellular space and 1% in ECF [72]. Though, Mg doesn't form an essential part of the hydroxyapatite lattice structure of the bone but it provides some support to bony structure at lattice surface. Mg present in bone contributes a little to its plasma pool. Plasma Mg usually exist in 3 forms: free Mg^{2+} ion (55%); plasma protein bound (30%) and complexed to phosphate and oxalate (15%) [72].

2. **Regulation of serum concentration:** Serum Mg concentration is tightly regulated in the range of 1.5–2.8 mg/dL across all age groups even in preterm neonates [73]. Serum concentration is mainly regulated by PTH and calcitonin at level of kidneys and bones. The role of vitamin D in intestinal absorption is controversial. Increase in PTH in response to decreased serum Mg will **increase** release of Ca and Mg from bone and increase tubular Mg reabsorption in kidneys. On the contrary, as Mg^{2+}acts as a cofactor for adenylate cyclase enzyme responsible for PTH secretion, chronic decrease in serum Mg^{2+} can lead to decreased PTH concentration and unresponsiveness. Increase in calcitonin in response to increased Mg^{2+}concentration will lead to decreased release from bone and increased excretion from the kidneys [52]. Recently, transient receptor potential melastatin 6 (TRPM6), has been recognised as a transcellular transport protein specific for Mg^{2+} in both renal tubules and intestines regulated by 17β estradiol [74].
3. **Role of intestines:** Dietary sources contributes maximum to Mg stores in the body. The rate of absorption is more in term and preterm neonates (60–85%) as compared to adults (34–62%) [75]. The main site of absorption of Mg is terminal ileum because of its longer

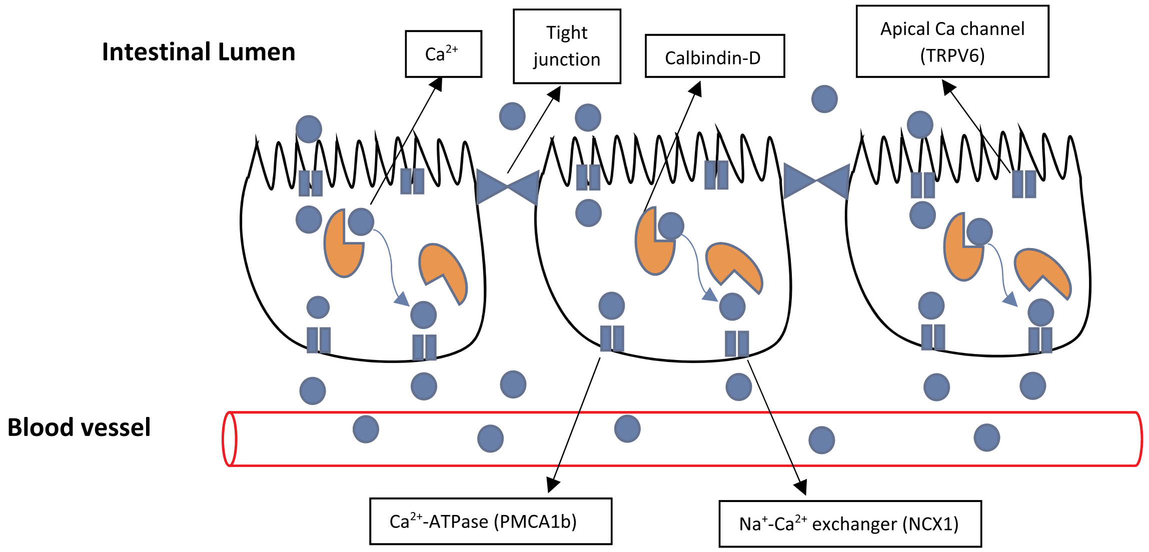
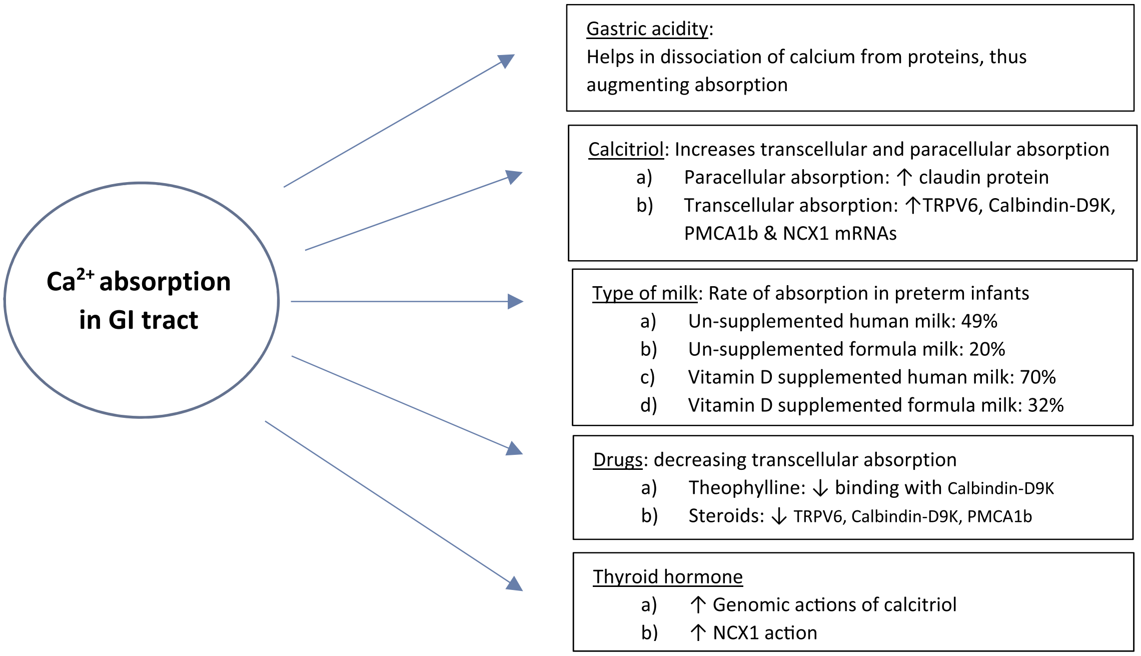


Fig. 2. Transcellular and paracellular pathway in GI tract.



Gastric acidity:
Helps in dissociation of calcium from proteins, thus augmenting absorption

Calcitriol: Increases transcellular and paracellular absorption
a) Paracellular absorption: ↑ claudin protein
b) Transcellular absorption: ↑TRPV6, Calbindin-D9K, PMCA1b & NCX1 mRNAs

Type of milk: Rate of absorption in preterm infants
a) Un-supplemented human milk: 49%
b) Un-supplemented formula milk: 20%
c) Vitamin D supplemented human milk: 70%
d) Vitamin D supplemented formula milk: 32%

Drugs: decreasing transcellular absorption
a) Theophylline: ↓ binding with Calbindin-D9K
b) Steroids: ↓ TRPV6, Calbindin-D9K, PMCA1b

Thyroid hormone
a) ↑ Genomic actions of calcitriol
b) ↑ NCX1 action

Fig. 3. Factors affecting GI absorption of calcium in neonates.

transit time [76]. Both active transcellular and passive paracellular mechanisms exist for Mg absorption**.** Factors affecting intestinal Mg absorption are depicted in Fig. 7.

4. **Role of kidneys:** As with other minerals, fine tuning of serum Mg^{2+} is mainly done by the kidneys. Though, studies on renal Mg^{2+} reabsorption are limited, it has been shown that around 85% of the filtered Mg is reabsorbed in PCT and thick ascending loop of Henle and 5–15% in DCT [77]. Active transcellular absorption of Mg in DCT regulates final excretion of Mg from body. This transcellular absorption is dependent on dietary Mg and various hormones described above. With increase in serum Mg concentration, the active transcellular absorption is decreased and vice versa with decrease in serum concentration [78]. Factors affecting renal reabsorption of Mg^{2+} are depicted in Fig. 7.

3.4. Differences in mineral physiology between preterm and term neonates

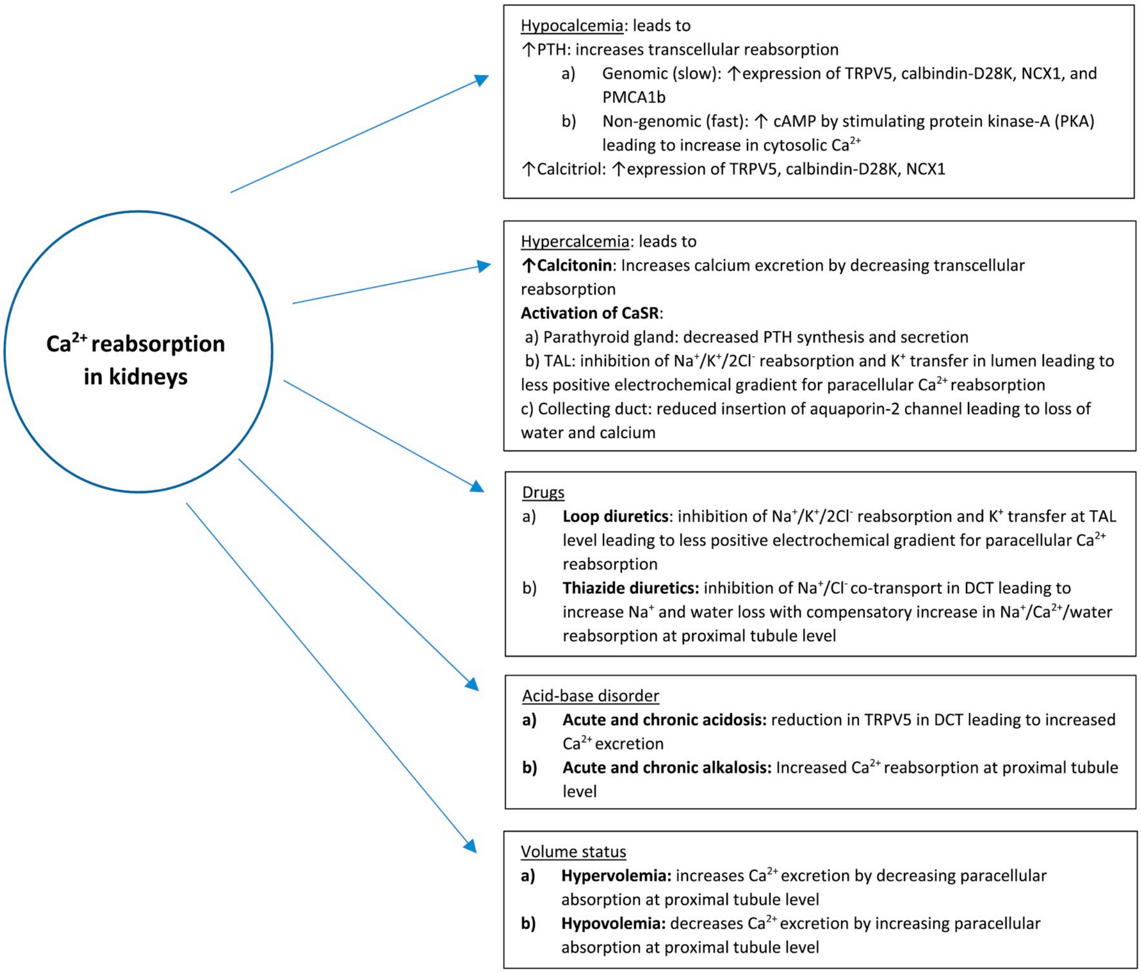
1. **Bone physiology:** Preterm neonates are born with a normal mineral content, however at a time, when they require high mineral accretion rate (120–200 mg/kg/day of Ca) [79]. This is usually met by placenta through active transport of **minerals** in-utero but if this requirement is not met in the extra-uterine life, it leads to compromised mineralization of skeleton thus leading to bone resorption to maintain serum mineral concentration. This is further aggravated due to immature intestines not capable of active absorption of Ca. Poor oral intake of Ca further compromises the Ca homeostasis, which results in a condition known as osteopenia of prematurity. In contrast, neonates born at term gestation accretes minerals at a much lower rate (30–40 mg/kg/day of Ca) which can be easily met by breast milk or formula milk [80].
2. **Intestinal absorption:** Intestines of preterm neonates exhibit a linear and non-saturable kinetics between intake and absorption, **consistent** with passive mechanism of absorption. This absorption is not affected by vitamin D intake, as has been shown in preterm infants who did not have any response to short courses of high calcitriol doses (0.1–3.0 μg/kg) [1,81]. It has been further suggested from studies in low birth weight infants that there is great variability in Ca retention (80–170 mg/kg/day) on the same preterm formula, possibly due to significant inter-subject variability in Ca bioavailability [82,83]. This may be in part due to endogenous fecal Ca excretion which may be quite variable (4–150 mg/day) in preterm infants [84]. With advancing postnatal and gestational age, intestines are able to retain Ca efficiently due to maturational change toward active, calcitriol-dependent, saturable mechanism of absorption [85]. Considering these factors, one must provide minerals parenterally in the first few days of life along with transition to oral feeds containing adequate amount of minerals (fortified human milk or special formula) to counter inherent limitations of mineral absorption in preterm infants.

Research points

- Mechanism of high serum Ca levels in foetus as compared to maternal serum
- Mechanisms involved in placental Pi and Mg transfer
- Pi and Mg homeostasis in preterm neonates
- Renal handling of minerals in preterm neonates

4. Summary

Mineral and bone physiology is considerably different in foetal and neonatal life, owing to different sources of minerals, mechanisms of transport, their regulation and requirements. In fetal life, placenta is the sole source of minerals, predominantly by active transport in last trimester, regulated by PTHrP and PTH. Foetal skeleton requires a high rate of mineral transport across placenta as it accretes most of the minerals in last trimester. After birth, in contrast, as placental support is lost, there is acute fall in serum Ca levels which take several days to normalise, possibly due to initial hypo-responsiveness of neonatal parathyroid function. The responsibility for providing minerals is taken over by intestines, deploying both mechanisms-active and passive transport. PTH, calcitriol, calcitonin, CaSR and FGF-23 start playing major roles in fine-tuning of serum mineral levels by affecting bone physiology, intestinal absorption and renal excretion. Preterm infants have difficulty retaining minerals due to immaturity of intestines which are non-responsive to calcitriol, leading to under mineralization of bones and osteopenia. However, most of our knowledge comes from animal studies, which may not be truly reflective of human physiology.



Hypocalcemia: leads to

↑PTH: increases transcellular reabsorption

a) Genomic (slow): ↑expression of TRPV5, calbindin-D28K, NCX1, and PMCA1b

b) Non-genomic (fast): ↑ cAMP by stimulating protein kinase-A (PKA) leading to increase in cytosolic Ca^{2+}

↑Calcitriol: ↑expression of TRPV5, calbindin-D28K, NCX1

Hypercalcemia: leads to

↑Calcitonin: Increases calcium excretion by decreasing transcellular reabsorption

Activation of CaSR:

a) Parathyroid gland: decreased PTH synthesis and secretion

b) TAL: inhibition of $Na^+/K^+/2Cl^-$ reabsorption and K^+ transfer in lumen leading to less positive electrochemical gradient for paracellular Ca^{2+} reabsorption

c) Collecting duct: reduced insertion of aquaporin-2 channel leading to loss of water and calcium

Drugs

a) **Loop diuretics**: inhibition of $Na^+/K^+/2Cl^-$ reabsorption and K^+ transfer at TAL level leading to less positive electrochemical gradient for paracellular Ca^{2+} reabsorption

b) **Thiazide diuretics:** inhibition of Na^+/Cl^- co-transport in DCT leading to increase Na^+ and water loss with compensatory increase in Na^+/Ca^{2+}/water reabsorption at proximal tubule level

Acid-base disorder

a) Acute and chronic acidosis: reduction in TRPV5 in DCT leading to increased Ca^{2+} excretion

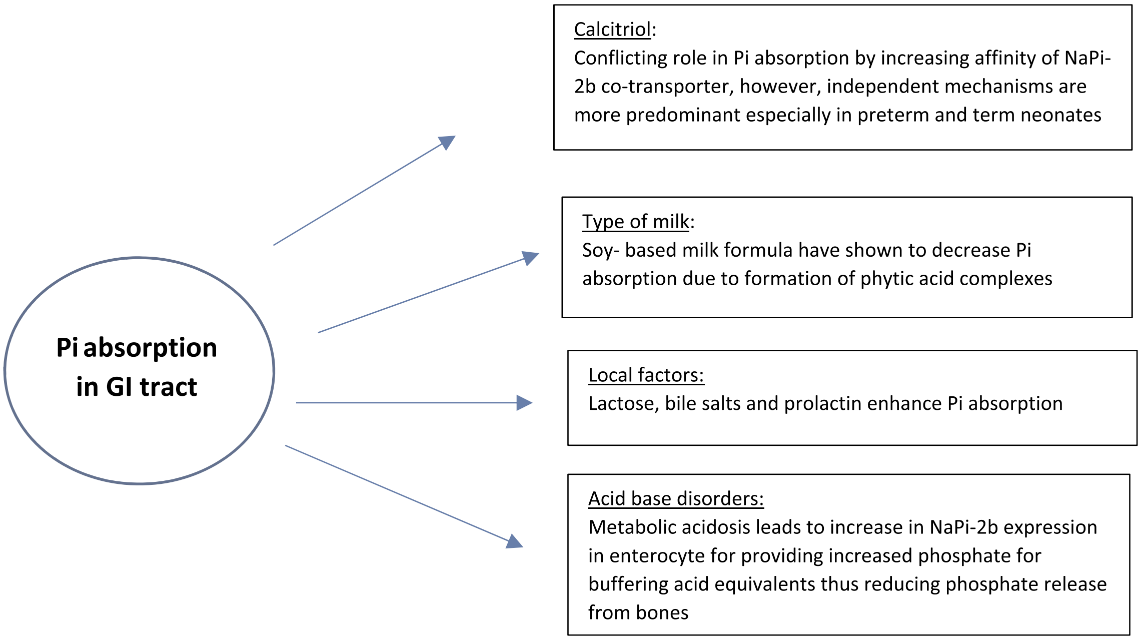
b) Acute and chronic alkalosis: Increased Ca^{2+} reabsorption at proximal tubule level

Volume status

a) Hypervolemia: increases Ca^{2+} excretion by decreasing paracellular absorption at proximal tubule level

b) Hypovolemia: decreases Ca^{2+} excretion by increasing paracellular absorption at proximal tubule level

Fig. 4. Factors affecting renal reabsorption of calcium in term and preterm neonates.



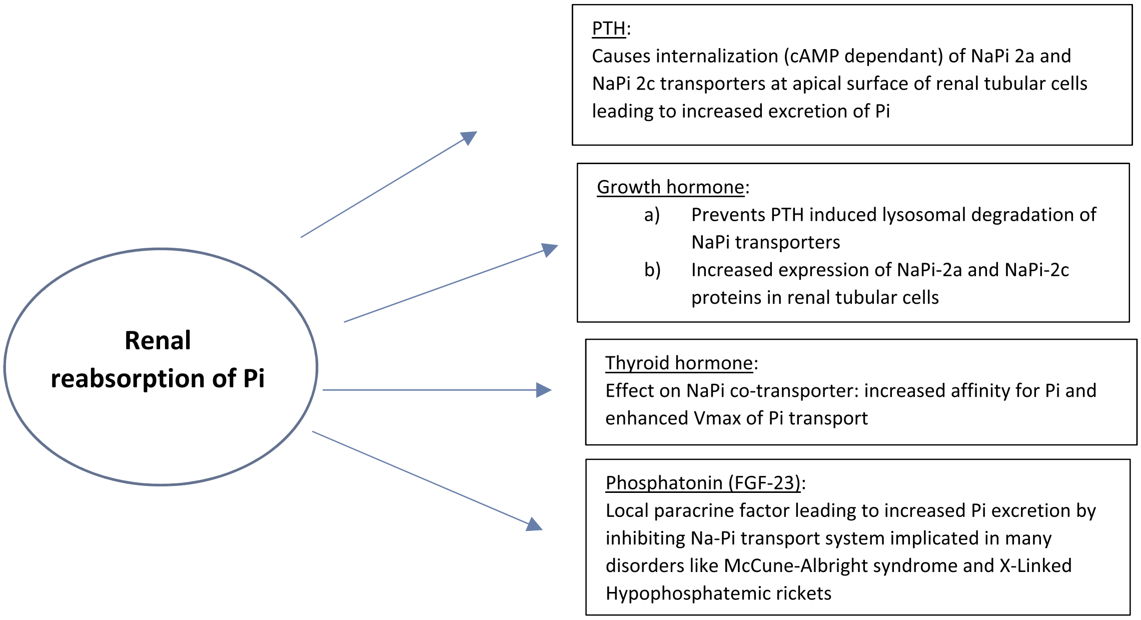
Calcitriol:
Conflicting role in Pi absorption by increasing affinity of NaPi-2b co-transporter, however, independent mechanisms are more predominant especially in preterm and term neonates

Type of milk:
Soy- based milk formula have shown to decrease Pi absorption due to formation of phytic acid complexes

Local factors:
Lactose, bile salts and prolactin enhance Pi absorption

Acid base disorders:
Metabolic acidosis leads to increase in NaPi-2b expression in enterocyte for providing increased phosphate for buffering acid equivalents thus reducing phosphate release from bones

Fig. 5. Factors affecting GI absorption of phosphorus.



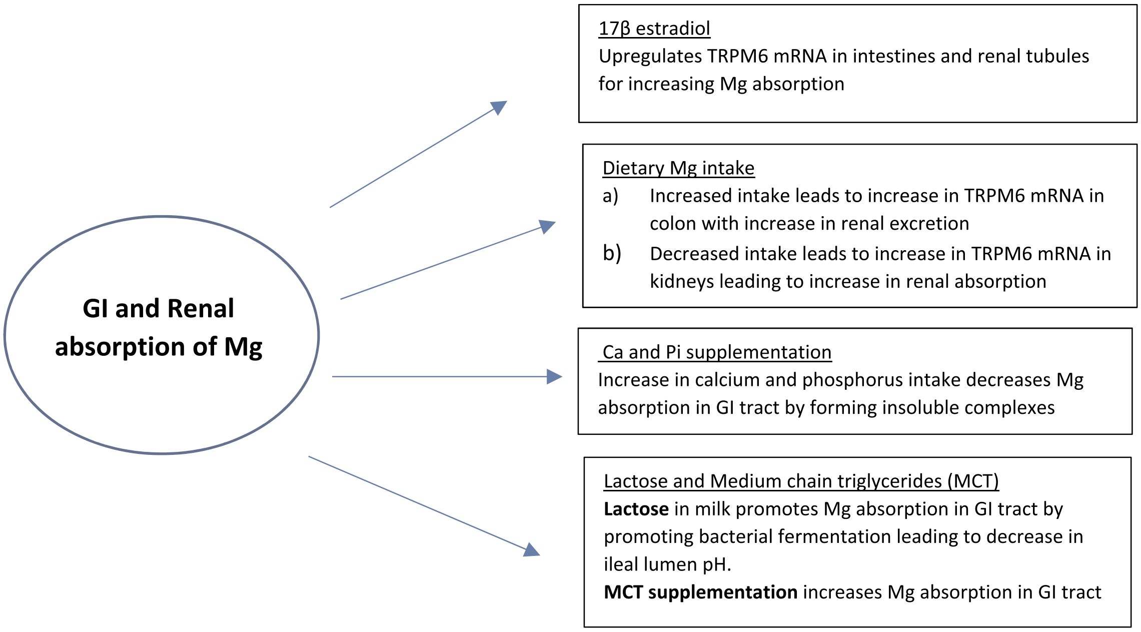
PTH:
Causes internalization (cAMP dependant) of NaPi 2a and NaPi 2c transporters at apical surface of renal tubular cells leading to increased excretion of Pi

Growth hormone:
a) Prevents PTH induced lysosomal degradation of NaPi transporters
b) Increased expression of NaPi-2a and NaPi-2c proteins in renal tubular cells

Thyroid hormone:
Effect on NaPi co-transporter: increased affinity for Pi and enhanced Vmax of Pi transport

Phosphatonin (FGF-23):
Local paracrine factor leading to increased Pi excretion by inhibiting Na-Pi transport system implicated in many disorders like McCune-Albright syndrome and X-Linked Hypophosphatemic rickets

Fig. 6. Factors affecting renal reabsorption of phosphorus (Pi) [69–71].



17β estradiol
Upregulates TRPM6 mRNA in intestines and renal tubules for increasing Mg absorption

Dietary Mg intake
a) Increased intake leads to increase in TRPM6 mRNA in colon with increase in renal excretion
b) Decreased intake leads to increase in TRPM6 mRNA in kidneys leading to increase in renal absorption

Ca and Pi supplementation
Increase in calcium and phosphorus intake decreases Mg absorption in GI tract by forming insoluble complexes

Lactose and Medium chain triglycerides (MCT)
Lactose in milk promotes Mg absorption in GI tract by promoting bacterial fermentation leading to decrease in ileal lumen pH.
MCT supplementation increases Mg absorption in GI tract

Fig. 7. Factors affecting GI and renal absorption of magnesium.

There are still gaps in knowledge regarding several aspects of mineral and bone physiology which remain to be elucidated in future.

Practice points

- Foetal life is characterized by higher serum Ca and Pi levels than postnatal life, with a high accretion rate in developing skeleton, mostly in the last trimester.
- Immediate postnatal life is characterized by fall in Ca and increase in Pi levels, reaching nadir at 24–48 h predisposing to symptomatic hypocalcemia in at risk infants.
- Maternal hypercalcemia may further aggravate postnatal fall in Ca levels by inducing parathyroid hypo-responsiveness in fetal life.
- Serum mineral levels normalise over several days to adult values, regulated by PTH, calcitriol, calcitonin and FGF-23 and dependant on enteral sources for minerals.
- Preterm birth predisposes infants to an immature passive intestinal transport mechanism, which, along with poor intake, cannot meet high mineral requirement of the growing skeleton, leading to osteopenia.
- With adequate nutrition, both parenteral and enteral, the needs of a preterm infant can be met and metabolic bone disease avoided.

Funding source

None.

Financial interests

None.

Declaration of competing interest

None.

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Seminars in Fetal and Neonatal Medicine 25 (2020) 101062

Contents lists available at ScienceDirect

Seminars in Fetal and Neonatal Medicine

journal homepage: www.elsevier.com/locate/siny

Calciotropic and phosphotropic hormones in fetal and neonatal bone development

Brittany A. Ryan, Christopher S. Kovacs[*]

Faculty of Medicine – Endocrinology, Memorial University of Newfoundland, St. John's, NL, Canada

A R T I C L E I N F O

Keywords:
Phosphorus
Calcium
Calcitriol
Fibroblast growth factor-23
Parathyroid hormone
Parathyroid hormone-related protein
Skeleton
Mineralization
Intestinal calcium absorption
Fetus
Neonate

A B S T R A C T

There are remarkable differences in bone and mineral metabolism between the fetus and adult. The fetal mineral supply is from active transport across the placenta. Calcium, phosphorus, and magnesium circulate at higher levels in the fetus compared to the mother. These high concentrations enable the skeleton to accrete required minerals before birth. Known key regulators in the adult include parathyroid hormone (PTH), calcitriol, fibroblast growth factor-23, calcitonin, and the sex steroids. But during fetal life, PTH plays a lesser role while the others appear to be unimportant. Instead, PTH-related protein (PTHrP) plays a critical role. After birth, serum calcium falls and phosphorus rises, which trigger an increase in PTH and a subsequent rise in calcitriol. The intestines become the main source of mineral supply while the kidneys reabsorb filtered minerals. This striking developmental switch is triggered by loss of the placenta, onset of breathing, and the drop in serum calcium.

1. Introduction

The regulation of bone and mineral homeostasis in the child and adult is dependent on key calciotropic and phosphotropic hormones, including parathyroid hormone (PTH), calcitriol, fibroblast growth factor-23 (FGF23), calcitonin, and the sex steroids (predominantly estradiol and testosterone). In fact, loss of any of these hormones can have adverse effects. This review will demonstrate that many of these hormones do not play the same key roles in the regulation of bone and mineral metabolism *in utero*. Instead, another hormone, PTH-related protein (PTHrP) plays an important role in the regulation of fetal bone and mineral metabolism, and still other relevant hormones remain to be identified.

Due to restrictions on the reference list, a comprehensive review will be principally cited that contains over 700 citations of the relevant primary literature [1].

2. Overview of fetal bone and mineral metabolism

The relative changes in calciotropic and phosphotropic hormones during fetal life are schematically shown in Fig. 1.

The fetal developmental period is characterized by rapid growth, mineralization, and high serum mineral concentrations. Unlike the adult, the intestines, kidneys and skeleton are not dominant sources of mineral during fetal development. Instead, the placenta must supply the minerals necessary to successfully mineralize the skeleton before birth. It does this by actively transporting calcium, phosphorus, and magnesium from the maternal circulation (Fig. 2). Placental transport of calcium and possibly magnesium has been shown to be regulated by PTHrP, although parathyroid hormone (PTH) may also play a small role [1]. During the latter part of pregnancy, when active placental mineral transport occurs, there is a considerable upregulation in the expression of genes involved in calcium transport. In turn, the fetal skeleton accretes about 80% of the 30 g of calcium, 20 g of phosphorus, and 0.8 g of magnesium it needs during the third trimester [1].

Serum mineral concentrations are maintained at higher levels in the fetus as compared to the mother in order to facilitate mineralization of the developing skeleton [1]. As early as 15 weeks of gestation (the earliest available data), human fetuses maintain serum and ionized calcium values about 0.30–0.50 mmol/L above the maternal levels [1]. Serum phosphorus and magnesium are maintained approximately 0.5 mmol/L and 0.05 mmol/L higher than in the mother, respectively [1]. In the adult, such high levels of calcium and phosphorus (calcium x phosphorus product) have unfavorable consequences from the formation of spontaneous calcium-phosphate crystals, which lead to soft tissue calcifications, calciphylaxis, coronary artery calcifications, and increased mortality. However, these high mineral concentrations are non-hazardous *in utero*, likely due to their rapid uptake into the

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[*] Corresponding author. Health Sciences Centre, 300 Prince Philip Drive, St. John's, NL, A1B 3V6, Canada.
E-mail address: ckovacs@mun.ca (C.S. Kovacs).

https://doi.org/10.1016/j.siny.2019.101062

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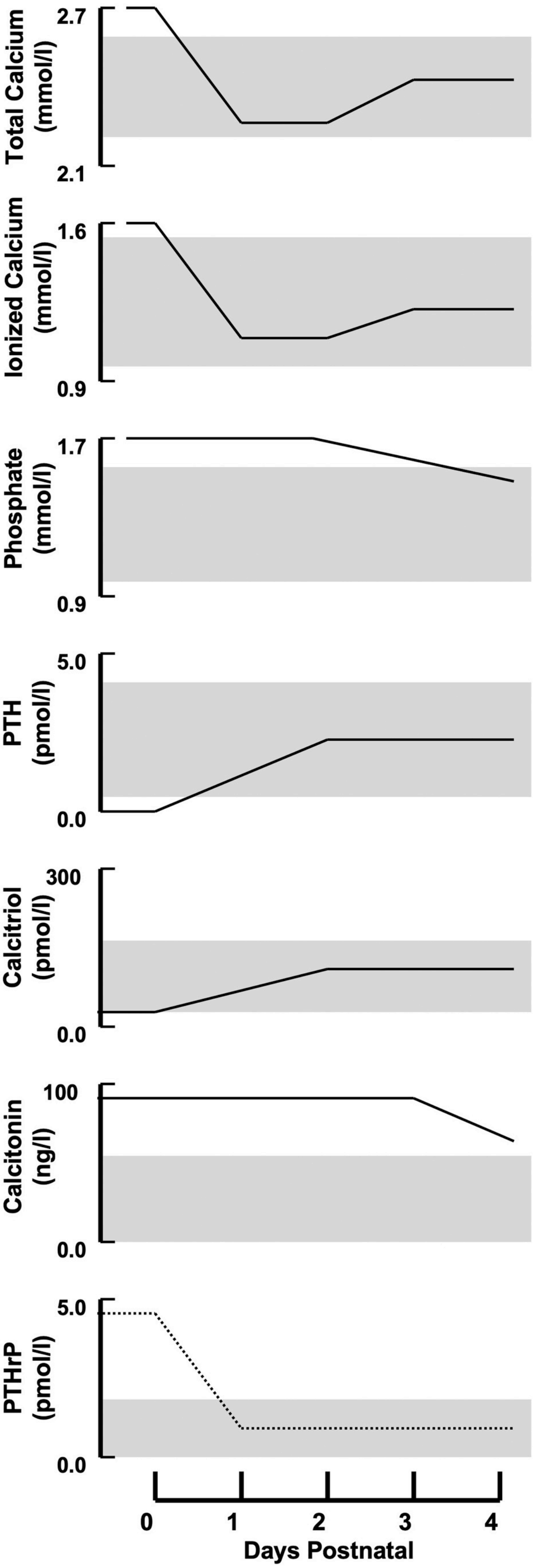

Fig. 1. Longitudinal changes in minerals and calciotropic hormones during fetal and neonatal development in humans. Normal adult ranges are indicated by the shaded areas. The progression in PTHrP has been depicted by a dashed line to reflect that it is speculative. FGF23 is not depicted because of the lack of data. Reproduced with permission ©1997 The Endocrine Society [63].

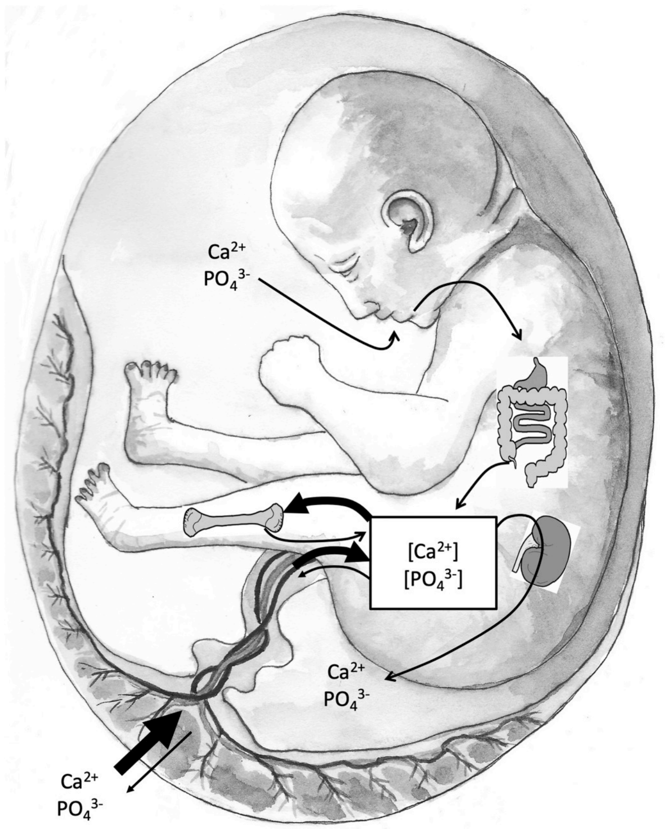

Fig. 2. Sources of minerals during fetal development. The main flux of calcium and phosphate is across the placenta and through the fetal circulation into bone; however, some mineral returns to the maternal circulation (backflux). The fetal kidneys filter the blood with little or no active reabsorption of minerals, such that the excretion of calcium is proportionate to its serum concentrations. Amniotic fluid, which is largely composed of fetal urine, is swallowed and absorbed, thereby restoring minerals to the circulation. However, this renal-amniotic-intestinal loop is likely a minor component for fetal mineral homeostasis. Although the net flux of minerals is into bone, some minerals are resorbed from the developing skeleton to reenter the fetal circulation and support the blood concentrations. For example, if placental delivery of calcium is deficient, fetal secondary hyperparathyroidism ensues, which resorbs calcium from the fetal skeleton, reduces skeletal mineral content, and may cause fractures *in utero* or during delivery.

skeleton and the relatively short duration of gestation.

Animal studies have shown that the high fetal ionized calcium is not set by the calcium-sensing receptor (CaSR); instead, the high calcium level acts through the CaSR to suppress PTH [1]. This is another difference between the fetus and the adult, because in the adult, the parathyroid CaSR sets the calcium level.

2.1. The role of calciotropic and phosphotropic hormones in the fetus

1. Parathyroid hormone

In the human baby, production of PTH becomes apparent at week 10 of gestation, although cord blood levels are significantly lower than in the mother [1]. As previously mentioned, this is because the high fetal ionized calcium acts through the CaSR to suppress PTH. Despite this suppression, release of fetal PTH can be stimulated by acute or chronic (e.g., from hypoparathyroidism) maternal hypocalcemia. Maternal PTH does not cross the placenta.

Studies of fetal mice, rats, and lambs indicate that absence of PTH causes hypocalcemia, hyperphosphatemia, low amniotic fluid mineral content, and reduced skeletal mineral content (in contrast, absence of PTH in the adult leads to increased renal calcium excretion and normal to increased skeletal mineral content) [1–3]. These findings suggest that, despite its low circulating levels, PTH is still required to achieve normal mineralization of the skeleton prior to birth. However, endochondral bone development in the absence of PTH is largely normal except for reduced mineral content [1–4], which suggests that the main role of PTH in fetal bone metabolism is to maintain the serum calcium. These data predict that aparathyroid human babies will be hypocalcemic at birth with low skeletal mineral content.

2. Parathyroid hormone related protein

Human cord blood shows elevated PTH-like bioactivity but low immunoreactive PTH [1]. This can be explained by PTHrP, which circulates at a concentration up to 15x greater than PTH [1]. PTHrP is also expressed in diverse fetal tissues including the placenta and fetal kidneys. Whether PTHrP is expressed in human fetal parathyroids is unknown, and reports from animal models are contradictory. However, since there is expression of PTHrP in adult parathyroids, it is conceivable that the fetal parathyroids express it as well. Maternal PTHrP cannot cross the placenta.

Much of what is currently known about the role of PTHrP in fetal bone metabolism comes from the study of *Pthrp* null fetal mice, which have accelerated endochondral bone development leading to shortened limbs and mandibles, domed skulls, and premature mineralization of bone [5]. They also display hypocalcemia, hypophosphatemia, and increased PTH [3,6,7]. The increase in PTH partly compensates for lack of PTHrP, since absence of both hormones leads to more marked hypocalcemia [3]. Conversely, although hypocalcemia occurs when PTH is absent, there is no compensatory increase in PTHrP, which indicates that PTHrP is not responsive to changes in fetal serum calcium [2].

Overall, it is clear that PTH and PTHrP both play roles in fetal bone and mineral homeostasis, since the absence of either hormone leads to adverse effects.

3. Calcitriol

Although 25-hydroxyvitamin D (25OHD) and calcitriol cross human and rodent placentas from mother to fetus, calcitriol typically circulates at less than 50% of the maternal value in the fetal circulation [1]. Calcitriol's concentration is greater in the human umbilical artery than vein, which suggests that the fetal kidneys and not the placenta are its dominant source. This has been confirmed by fetal nephrectomy in lambs, which lowers the circulating level of calcitriol [1]. Moreover, the fetal kidneys are capable of upregulating calcitriol's synthesis in response to such factors as absence of the vitamin D receptor (*Vdr* null fetuses) [8], and increased PTH (*Casr* null fetuses) [9].

The placenta expresses 1α-hydroxylase (CYP27B1), which converts 25OHD to calcitriol, and 24-hydroxylase (CYP24A1), which catabolizes 25OHD and calcitriol into inactive forms [1].

During pregnancy, calcitriol more than doubles in the maternal circulation, and the placenta has often been assumed to be responsible. However, an anepheric woman on dialysis had low endogenous calcitriol before, during, and after pregnancy [10]. Recent studies of *Cyp27b1* null mice have shown that the placenta contributes at most a small amount of calcitriol to the maternal circulation [11,12]. Conversely, some maternal calcitriol contributes to the fetal circulating level [12]. Overall, the human and animal data indicate that the maternal kidneys are responsible for the marked increase in maternal calcitriol during pregnancy.

Calcitriol synthesis in the fetus is suppressed by the high circulating concentrations of calcium and phosphorus, and low concentrations of PTH [1]. Further to this, placental expression of CYP24A1 contributes to preferential catabolism of 25OHD and calcitriol into their 24-hydroxylated metabolites [1].

Despite playing a critical role in bone and mineral metabolism in both the child and the adult, evidence from both animal models and human disorders indicate that loss of fetal calcitriol or the VDR is not detrimental to fetal mineral homeostasis and skeletal development. Studies of severely vitamin D deficient rats [13–16], *Cyp27b1* null pigs [17,18], *Cyp27b1* null mice [12], and *Vdr* null mice [8,19], have consistently shown normal fetal serum calcium, phosphorus, PTH, and skeletal morphology, ash weight, and mineral content. Observational studies in human babies have shown that severe vitamin D deficiency does not alter fetal serum calcium, phosphorus, PTH, and skeletal morphology, ash weight, or mineral content [1]. Human babies with inactivating mutations of *VDR* or *CYP27B1* are also normal at birth [1].

Multiple randomized clinical interventions have tested whether maternal vitamin D supplementation during pregnancy has any beneficial effects on the fetus. Individual studies that involved women with severe vitamin D deficiency [20–22], as well as meta-analyses and reviews of all studies [1,23–25], have shown that increased fetal 25OHD does not alter cord blood calcium, phosphorus, or PTH; nor does it affect skeletal parameters at birth.

Why does loss of calcitriol or its receptor not affect fetal mineral homeostasis or skeletal development? Quite simply, the placenta is the source of minerals and it does not require calcitriol for this function. It is after birth and loss of the placental mineral infusion that absence of calcitriol begins to have effects. This is because calcitriol's main role with respect to the skeleton is indirect, through its stimulation of intestinal calcium and phosphorus absorption.

4. Fibroblast growth factor 23

FGF23, a phosphorus regulating hormone, is produced largely by osteoblasts and osteocytes in fetal bone. There have been few studies of FGF23 levels in healthy human babies, with the available data demonstrating intact fetal FGF23 levels to range from low to equal to the maternal value [1,26,27]. On the other hand, C-terminal FGF23 has been found to be up to 2x higher than maternal values [1,26,27]. These data suggest that there is plentiful FGF23 in the fetal circulation, which is rapidly cleaved, leaving an abundance of non-functional C-terminal fragments.

Studies of animal models have shown that absence of FGF23 or its co-receptor Klotho, and 10-fold or higher levels of FGF23, do not alter serum and amniotic fluid phosphorus, PTH, skeletal mineral content and morphology, and placental phosphorus transport [28,29]. Maternal FGF23 does not cross the placenta. These findings indicate that FGF23 is not an important regulator of fetal phosphorus homeostasis.

5. Calcitonin

The C-cells of the thyroid differentiate at approximately week 12 of gestation and calcitonin is detectable in those cells by the 15th week of gestation [1]. In both term and pre-term babies, serum calcitonin levels are double that of their mothers [1]. Calcitonin is also expressed by trophoblasts of the placenta, which may also contribute to the circulating calcitonin levels in the fetus. Loss of calcitonin and calcitonin gene-related peptide-alpha (*Ctcgrp* null mice) cause hypomagnesemia and reduced skeletal magnesium content, but no disturbance in serum and amniotic fluid calcium, PTH, skeletal mineral content and morphology, and placental calcium transport [30]. Maternal calcitonin does not cross the placenta [1]. These findings indicate that calcitonin is not a key regulator of calcium or phosphorus metabolism, but may be important in fetal magnesium homeostasis.

6. Sex Steroids

As early as week 5 of gestation, pituitary cells produce two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) [1]. These circulate at higher concentrations in female fetuses throughout gestation [1]. LH, FSH and chorionic gonadotropin (derived from the placenta) act on the Leydig cells within the male testes to produce testosterone [1]. The concentration of testosterone in males peaks during embryonic weeks 11–17, and then declines until term, at which time testosterone levels are not significantly different between males and females [1]. The ovaries, testes and adrenal glands don't produce estradiol until later in gestation. At term, there is also little difference between estradiol concentrations of male and female babies. The concentrations of both estradiol and testosterone at term are significantly lower than maternal values [1].

A human baby lacking estrogen receptor alpha had normal birth weight and length [31]. Mice lacking estrogen receptor alpha or beta are normal at birth and until at least three weeks of age [1]. These limited data imply that estradiol and testosterone do not contribute to fetal mineral homeostasis or skeletal development, but specific fetal data are lacking.

2.2. *Role of the fetal parathyroids*

In both humans and mice, the parathyroids and thymus have a common developmental origin within the third pharyngeal pouch [1]. A second pair of parathyroids originate from the fourth pharyngeal pouch in humans; however, mice lack this second pair [1,32]. The developing parathyroids are fragile, which can lead to their fragmentation during embryonic migration, with parathyroid tissue being found in the thymus and other ectopic regions [1].

The fetal parathyroids contribute to calcium and phosphorus homeostasis by secretion of both PTH and PTHrP. This has been made clear through studies of animal models, which have shown that loss of parathyroids, PTH, or PTHrP, each lead to hypocalcemia and hyperphosphatemia with reduced skeletal mineralization [2,3,6]. When both PTH and PTHrP, or the PTH receptor, are lacking, more pronounced hypocalemia results with a further reduction in skeletal mineral content [3,6,7].

When maternal hypocalcemia occurs, the fetal parathyroids have the ability to secrete higher levels of PTH. This will maintain a normal serum calcium in the fetus, but can lead to hyperplasia of the parathyroids, skeletal resorption, loss of mineral content, and fractures occurring *in utero* or during delivery [1]. In contrast, maternal hypercalemia results in increased placental calcium transport and supression of the fetal parathyroids. This may have negative impacts after birth, with hypoparathroidism of the neonate that can be prolonged or permanent [1].

2.3. *Renal mineral absorption and the amniotic fluid*

What is known about the role of fetal kidneys in the regulation of bone and mineral metabolism has largely come from study of animal models. There have been no measurements of serum mineral or calciotropic hormones from anephric human fetuses, but they have exhibited skeletal anomalies (scoliosis, rib anomalies, and absent toes, radii or thumbs) [1]. Whether these anomalies result from the underlying genetic disturbances or absent kidney function is unknown. Amniotic fluid is scant without fetal kidneys, confirming that it largely consists of urine.

Studies of animal models suggest that the fetal kidneys play a minor role in mineral metabolism, because the placenta is the dominant organ of mineral and fluid exchange. Moreover, any minerals excreted by the fetal kidneys are not lost because they enter the amniotic fluid, which is then swallowed, absorbed, and brought back into the fetal circulation (Fig. 2).

2.4. *Fetal intestinal mineral absorption*

The fetal intestines play a minor role in mineral metabolism because the placenta is the main supplier of minerals [1]. Although it is not possible to study the function of human fetal intestines, study of preterm neonates (the functional equivalent of fetuses) allow us to infer the function of the fetal intestines. Preterm babies show intestinal absorption of calcium that is proportional to calcium intake and does not reach a maximum, suggesting that passive absorption is the main method of intestinal calcium absorption in the fetus [1]. The calcium requirement of the preterm neonate is very high at birth because the fetal skeleton is typically accreting 120–150 mg/kg of calcium daily at that age, which the preterm neonate's intestines cannot absorb [1]. Current guidelines recommend a daily calcium intake of 120–200 mg/kg for preterm babies, which requires special oral formulas and parenteral feeding [1].

Although the fetal intestines are a minor player in mineral homeostasis (Fig. 2), they do impact amniotic fluid volume. When swallowing or absorption of amniotic fluid cannot occur, such as from blockages in the upper or lower gastrointestinal tract, there is increased amniotic fluid volume (polyhydramnios).

2.5. *Placental mineral transport*

The placenta plays a critical role to pump calcium and other minerals from the maternal circulation. This may occur by simple diffusion, paracellular transport, vesicular transport, or active transport [1,33].

Active transport of minerals across the placenta is necessary for the fetus to meet its requirements. A study which perfused human placentas in vitro determined that approximately one third of the forward flow of calcium from mother to fetus occurs via active transport [34]. It is thought that active placental calcium transport occurs using a similar mechanism to intestinal calcium transport, in which calcium enters trophoblasts through gated channels such as TRPV6, is shuttled across the cell interior bound to proteins such as calbindin D-9k, and is actively pumped out by Ca^{2+}-ATPase [1].

It is not possible to directly measure placental mineral transport in human fetuses. However, indirect measurements suggest that the ambient maternal blood calcium influences the rate of placental calcium transport [1]. As mentioned previously, maternal hypercalcemia increases the flow of calcium from mother to fetus, causing a suppression of the fetal parathyroids. Conversely, maternal hypocalcemia reduces the supply of calcium to the fetus, and has been associated with compensatory fetal hyperparathyroidism [1]. Animal data also support that the mechanism of placental calcium transport is upregulated in the presence of maternal hypocalcemia, such that an intravenous bolus of calcium in the maternal circulation causes a marked increase in the fetal serum calcium that is not seen in fetuses of normocalcemic mothers [1].

Several lines of evidence suggest that PTHrP is an important regulator of active placental calcium transport. In studies of fetal lambs, rats and sheep, placental calcium transport has been shown to be stimulated by fragments of PTHrP that encompass the mid-region of the molecule [1]. Moreover, *Pthrp* null fetuses have a lower rate of calcium transport across the placenta which can also be corrected by treatment with mid-molecular PTHrP [6]. This form of PTHrP has no similarity to PTH and cannot bind to or activate the PTH receptor [1].

There is some evidence that PTH may play a small effect in stimulating placental calcium transport, which has been most clearly elucidated in *Pth* null fetuses [2].

Much less is known about the regulation of placental phosphorus and magnesium transport. Study of fetal lambs have demonstrated that mid-molecular PTHrP stimulates active magnesium transport whereas PTH does not, while none of PTH, PTHrP, calcitriol, or FGF23 regulate placental phosphorus transport [1].

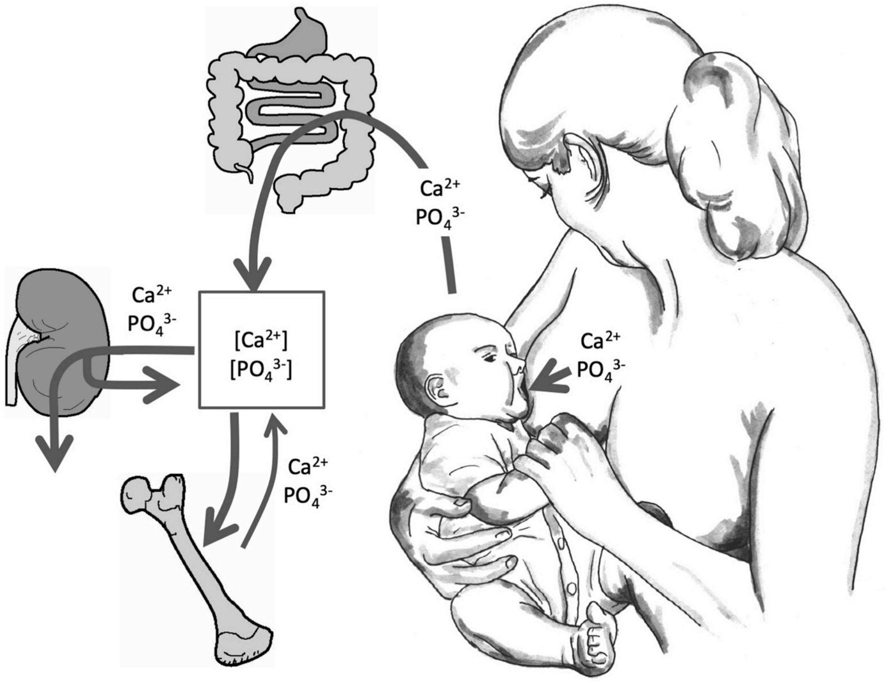

Fig. 3. Sources of minerals in the neonate. Loss of the placenta leads to the intestines and kidneys becoming the main sources of minerals for the neonate. The lactose content of milk facilitates calcium absorption, whereas the high phosphate content of formula leads to lower calcium absorption as compared to breastmilk. The kidneys begin to actively reabsorb calcium and phosphate after birth. Ongoing skeletal resorption contributes to maintaining the serum concentrations of minerals, although the main flux of minerals is into the developing skeleton.

3. Overview of neonatal bone and mineral metabolism

The relative changes in calciotropic and phosphotropic hormones during neonatal life are also schematically shown in Fig. 1.

After birth, there are significant changes in mineral physiology. Serum and ionized calcium typically decrease by 20–30% in the first 12–24 h; by several days after birth, calcium levels have risen to that of a normal adult [1]. Phosphorus levels increase over the first 24–48 h and then, like calcium, decrease to match adult values by several days post birth [1].

While the fetal intestines played a negligible role in fetal mineral metabolism, the intestines become the main source of minerals in the neonate. Meanwhile, the kidneys begin to reabsorb mineral, and bone turnover contributes to circulating mineral concentrations (Fig. 3). These changes are provoked by loss of the placenta and the onset of breathing, which respectively cause loss of calcium inflow and a rise in pH. In turn these cause the decline in blood ionized calcium after birth, which is followed by an increase in PTH and then calcitriol. FGF23 begins to regulate serum phosphorus, renal phosphorus excretion, and the synthesis and catabolism of calcitriol. Intestinal calcium absorption is initially passive, facilitated by lactose in the mother's milk, and switches to active transport requiring calcitriol.

3.1. Role of calciotropic and phosphotropic hormones in the neonate

1. Parathyroid hormone

The abrupt fall in ionized calcium leads to upregulation of PTH synthesis and release in the hours after birth. PTH remains low during the first 12–24 h and peaks at approximately 48 h. This spike in PTH stimulates CYP27B1 to form active calcitriol, and inhibits the catabolic enzyme CYP24A1 [35,36].

2. Parathyroid hormone-related protein

Presumably loss of placental PTHrP leads to a fall in its circulating concentration after birth, but data to confirm this are lacking. In the neonate, infant, and child, PTHrP continues to be produced by chondrocytes and osteoblasts, and to play a critical role in endochondral bone development. High concentrations of PTHrP are found in milk, but not in infant formula. The exact role of PTHrP in mother's milk is unknown; however, some data suggest that it may play a role in neonatal intestinal calcium absorption or skeletal accretion of mineral [37].

3. Calcitriol

By 48 h after birth, circulating calcitriol in the human neonate increases to adult values as a result of the spike in PTH levels. In neonatal rodents and lambs, a high level of Cyp24a1 activity keeps the concentration of calcitriol low until 2–3 weeks after birth, even when treated with pharmacological doses of PTH [38–40]. This suggests that the neonate suppresses calcitriol formation until the intestines are developmentally ready to respond to calcitriol.

Calcitriol's role to stimulate active intestinal calcium and phosphate absorption becomes much more critical in the weeks and months after birth. Severe vitamin D deficiency can present with hypocalcemia as early as 48 h after birth; conversely, randomized trials have shown that prenatal vitamin D supplementation prevents this [20,21]. Vitamin D deficiency leading to hypocalcemia or skeletal rickets is typically not seen until weeks after birth, with peak incidence at age 6–18 months [41–43]. This is likely because intestinal calcium transport remains passive postnatally until the intestines mature enough to accommodate calcitriol-dependent active calcium transport. Children who lack CYP27B1 (pseudovitamin D deficiency; PDDR) or functional VDRs (hereditary vitamin D resistant rickets; HVDDR), are also normal at birth. However, by age 2–8 months in children with PDDR, and by 1–2 years in children with HVDDR, hypocalcemia, hypophosphatemia and rickets ensue.

4. Fibroblast growth factor 23

From the scant human data available, it appears that intact FGF23 increases markedly after birth [44,45], while C-terminal FGF23 stays the same as fetal values [44]. It is unclear exactly when FGF23 becomes important in regulating postnatal phosphorus metabolism; however, individual case reports have documented how early deficiency or excess FGF23 become evident. Hereditary hyperphosphatemic tumor calcinosis due to loss of FGF23 has presented with a calcified mass as early as 18 days after birth [46,47]. X-linked hypophosphatemic rickets, in which mutations in *PHEX* cause high levels of FGF23, has led to hypophosphatemia and hyperphosphaturia as early as 9 days after birth [48]. However, most affected individuals with tumor calcinosis or XLH are not recognized until much later in childhood or as adults [1].

Mice lacking FGF23 or its co-receptor Klotho develop hyperphosphatemia with reduced renal phosphorus excretion by 4–5 days after birth [28]. This leads to high levels of calcitriol, marked hypercalcemia, soft tissue calcifications, skeletal deformities, and death by 10–12 weeks of age [49]. In contrast, excess FGF23 in *Phex* null mice causes hypophosphatemia and increased renal phosphorus excretion by 12 h after birth, and by 72 h the bones are shorter and under-developed compared to their WT counterparts [28,29].

5. Calcitonin

Calcitonin increases 10-fold during the first 48 h after birth before it gradually declines to the low levels of the adult [1]. Study of human babies suggest that calcitonin may have little importance in bone and mineral homeostasis. While data from early studies suggested that higher calcitonin levels are correlated with hypocalcemia in the preterm neonate, later studies showed that gestational age at birth is a more significant predictor of calcitonin levels [1].

6. Sex Steroids

Estradiol remains low after birth in both male and female neonates, and stays that way until the onset of puberty [1]. Likewise, testosterone remains very low in females, but spikes in males during the first 24 h and again during the first several months of life, after which it returns to low levels until onset of puberty [1]. Although both estradiol and testosterone play a critical role in bone mineral metabolism in the adult, there is no evidence to support a role in the neonate [1].

3.2. The role of the neonatal parathyroids

Consistent with suppression of the parathyroids during fetal life, in the first 48 h after birth the parathyroids are slow to respond to the drop in ionized calcium [50–53]. After this, the parathyroids become responsive and act to maintain the serum ionized calcium [50,51].

A similar increasing responsiveness of the parathyroids is evident in animal models. In fact neonatal rodents that have had their parathyroids removed show normal bone structure and mineral content [54]. This is consistent with PTH playing no role in early neonatal bone and mineral homeostasis, during which mineral absorption is passive and facilitated by the lactose content of milk. Absence of parathyroids is also not critical in humans, since less than 50% of individuals with congenital hypoparathyroidism present with hypocalcemia in childhood [1].

3.3. The role of the neonatal kidneys

Despite high cord blood calcium and low PTH at birth, which would cause hypercalciuria in an adult, renal calcium excretion is low in the neonate over the first week. This is due to low renal blood flow and glomerular filtration rate, in conjunction with low renal expression of VDR and CaSR [1]. A subsequent developmental increase in renal blood flow, glomerular filtration rate, and expression of the VDR and CaSR, contribute to a progressive increase in renal calcium excretion, despite lower serum ionized calcium and higher PTH in the neonate at this stage [1].

Phosphorus excretion is also low at birth but subsequently increases [55,56]. This is likely due to both the increasing level of PTH in the circulation and a developmental increase in renal responsiveness to PTH, which have been demonstrated in term and preterm infants [55,57].

The rise in PTH after birth upregulates renal CYP27B1, which leads to an increase in calcitriol.

3.4. Intestinal mineral absorption in the neonate

As previously noted, pre-term human neonates show passive, non-saturable intestinal calcium absorption that does not require calcitriol [1]. The high lactose content found in breast milk increases passive calcium absorption and net dietary calcium absorption [1]. Studies of newborn rodents have demonstrated that with increasing gestational and postnatal age, the expression of VDR, calbindin D-9k, and TRPV6 upregulate within intestinal cells, enabling calcitriol-dependent active calcium absorption to onset [1].

Phosphorus is also absorbed through passive and active pathways. The mechanism of passive phosphorus transport mirrors that of passive calcium transport, however, the mechanism of active phosphorus transport differs. Phosphorus is actively transported through the intestine via the sodium phosphate transporter NAPI2B, which is regulated by calcitriol, phosphorus intake and FGF23 [58].

The ability of the neonate to absorb calcium depends on the form of nutrition that the baby receives. Intestinal absorption of calcium is more efficient in breastfed infants in comparison to those fed with formula [59–61]. In contrast, the high phosphate content of formulas can lead to a doubling of serum phosphorus as compared to breast-fed neonates [59,61,62]. These nutritional differences can have a significant impact on the developing baby. Breast-fed babies have higher serum calcium and lower serum phosphorus, and therefore a lower risk of hypocalcemia than those who are formula-fed [59,62].

4. Conclusions

Skeletal development in the fetus and the neonate are dependent on adequate mineral delivery to allow for mineralization of bone. During fetal development, the placenta is the main source of minerals. Placental mineral transport is regulated by PTHrP and possibly PTH, but not by calcitriol, FGF23, calcitonin, or the sex steroids. It is after birth that the intestines become the key route of mineral delivery, and dependent upon calcitriol to stimulate active calcium absorption. FGF23 begins to play a critical role in the postnatal kidneys to regulate phosphorus metabolism. Consequently, disorders of vitamin D/calcitriol or FGF23 do not affect the fetus but can lead to disordered mineral and bone metabolism in the neonate and infant.

Practice points

- absence of PTH will lead to fetal hypocalcemia and an undermineralized skeleton
- absence of calcitriol or VDR does not disturb mineral homeostasis or skeletal development until after birth
- disorders of FGF23 affect phosphorus physiology only after birth
- PTHrP is an important regulator of fetal mineral homeostasis and skeletal development

Research directions

- why does FGF23 excess or deficiency not affect fetal phosphorus metabolism?
- what factors control placental mineral transport?
- are there sensors that set the fetal blood calcium and phosphorus concentrations?

Acknowledgement

Canadian Institutes of Health Research (#133413 and #419194); Faculty of Medicine and Discipline of Medicine, Memorial University [to CSK].

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Seminars in Fetal and Neonatal Medicine 25 (2020) 101077

Contents lists available at ScienceDirect

Seminars in Fetal and Neonatal Medicine

journal homepage: www.elsevier.com/locate/siny

Post-natal bone physiology

Rania Ali El-Farrash[a,*], Radwa Hassan Ali[b], Noha Mokhtar Barakat[a]

[a] *Department of Pediatrics, Faculty of Medicine, Ain Shams University, Abbassya Square, 11566, Cairo, Egypt*
[b] *Physiology Department, Faculty of Medicine, Ain Shams University, Abbassya Square, 11566, Cairo, Egypt*

ARTICLE INFO

Keywords:
Bone cells
Osteoblast
Osteoclast
Bone remodeling
Mechanical stimulation
Bone growth

ABSTRACT

Post-natal bone development is characterized by substantial longitudinal bone growth and changes in skeletal size and shape. Bone is in a dynamic process of continuous remodeling which helps to regulate calcium homeostasis, repair micro-damage to bones from everyday stress, and to shape the skeleton during growth. Bone growth is regulated by systemic hormones and locally generated factors. Understanding their mechanisms of action enables us to obtain a better appreciation of the cellular and molecular basis of bone remodeling and could therefore be valuable in approaches to new therapies. This article will review molecular and cellular control of skeletal growth in the post-natal period, the physiology of each bone cell with their systemic and local regulators, as well as the physiology of bone remodeling.

1. Introduction

Post-natal bone development is characterized by substantial longitudinal bone growth and changes in skeletal size and shape. After birth, important post-natal adaptations of the skeletal system to extrauterine conditions occur [1]. These post-natal adaptations are regulated by a large variety of transcriptional, paracrine and endocrine signaling systems. In this chapter, we focus on chosen regulatory factors because of their established importance in normal human physiology and disease. Mechanical strain is one of the most powerful stimulators of bone formation and growth. A Recent study has demonstrated that range-of-motion exercises improve bone mineral content and post-natal bone growth [2]. The cellular and molecular control of skeletal development and growth will be discussed followed by some of the prime endocrine controlled regulatory systems of bone growth and remodeling.

2. Endochondral and intramembranous bone formation

Bone formation starts with the migration of mesenchymal cells to the sites of the future bones, where they differentiate either into chondrocytes to form cartilage template of the future bones (endochondral bone formation) or differentiate into osteoblasts to form bone directly without a cartilage mold (intramembranous bone formation) [3].

All the long bones of the axial skeleton (vertebrae and ribs) and the appendicular skeleton (limbs) develop and grow through endochondral ossification. Endochondral ossification is a complex process, in which bone formation begins from a cartilaginous template [4]. This is followed by vascular invasion of the template and replacement of the cartilage by bone through the actions of osteoblasts and osteoclasts [5]. Endochondral ossification begins with points in the cartilage called “primary ossification centers”. They mostly appear during fetal development between the 8th and 12th weeks, but most of the mineralization of bone does not occur until the third trimester. Secondary ossification centers form in the femurs by the 34th week, thereby creating true growth plates. Linear growth ceases when these growth plates fuse during puberty [6].

Intramembranous ossification is responsible for the formation of the flat bones of the skull, mandible, maxilla, and clavicles [1]. The development of these bones also starts with mesenchymal progenitor cells forming condensations at the sites where the bones will be formed [7]. Yet, in the mesenchymal condensations of intramembranous bones, cells do not differentiate into chondrocytes but instead differentiate directly into pre-osteoblasts and then osteoblasts that deposit “osteoid” or bone matrix rich in type I collagen. As the osteoblasts mature further, the bone matrix becomes progressively mineralized [8].

3. Cellular control of bone growth

3.1. Chondrocytes

The growth plate is formed of a resting zone which contains small round chondrocytes that are mitotically inert, these cells called stem-like or progenitor cells that require the activity of b-catenin for cell maintenance [9]. The next zone contains flattened, stacked chondrocytes that are mitotically active and form fairly regular columns.

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* Corresponding author.
E-mail addresses: rania.elfarrash@med.asu.edu.eg (R.A. El-Farrash), dr.radwahassan@med.asu.edu.eg (R.H. Ali), noha_mokhtar84@yahoo.com (N.M. Barakat).

https://doi.org/10.1016/j.siny.2019.101077

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The chondrocytes at the lower end of this zone will begin to enlarge, becoming first pre-hypertrophic and then hypertrophic chondrocytes (HCCs). Some of the HCCs will undergo apoptosis, while others differentiate into osteoblasts or other cells of the bone marrow cavity [10].

The transcription factor Sox9 is the main factor responsible for the conversion of progenitor cells in the mesenchymal condensations into chondrocytes, whereas factors Sox9, 5, and 6 combined action is required to direct the subsequent differentiation of chondrocytes throughout all phases of the chondrocyte lineage [11].

Parathyroid hormone-related peptide (*PTHrP*), *Indian hedgehog* (*Ihh*) and fibroblast growth factors (*FGFs*) genes are critical for the development and regulation of the cartilage growth plate [12].

The *Ihh* gene (2q35) is the key regulator of bone development; it coordinates chondrocyte proliferation, differentiation and osteoblast differentiation. During endochondral bone development, Ihh is synthesized by pre-hypertrophic chondrocytes and early HCCs. Ihh binds to its receptor Patched-1 (Ptc-1); which leads to activation of Smoothened (Smo), a membrane protein required for the cellular actions of Ihh. Then, active Smo triggers a cascade that leads to gene activation. Moreover, Ihh is responsible for conversion of perichondrial cells to osteoblasts [13].

PTHrP is a protein secreted during fetal life by perichondrial cells at the ends of cartilage molds and by early proliferative chondrocytes. PTHrP acts on the same receptor as parathyroid hormone (PTH). These PTH/PTHrP receptors (PPRs) are expressed at low levels by proliferating chondrocytes and at high levels by pre-hypertrophic and early HCCs under the effect of Ihh. PTHrP acts primarily to slow down the further chondrocyte differentiation to hypertrophy, so it keeps chondrocytes in the proliferative pool maintaining the adequate length of columns [14]. By preventing premature hypertrophic differentiation, Ihh production is consequently slowed down thus the production of PTHrP is lowered. PTHrP and Ihh regulate each other via a negative feedback pathway and both interact together to controls the rate of chondrocyte differentiation in the growth plate [15].

Studies have shown that *FGF* signaling crucially regulates chondrocyte proliferation and differentiation. Many of the 23 distinct *FGF* genes and the four *FGF receptors* genes (*FGFR1-4*) are expressed during endochondral bone formation (Table 1) [16]. Activating mutations in *FGFR3* gene result in chondrodysplasias and dwarfism while mutations in *FGFR1* or *2* genes cause craniosynostosis, which is due to a decrease in chondrocyte proliferation in the growth plate and increased apoptosis [17]. Moreover, in transgenic mice, activation of *FGFR3* leads to a decrease in the expression of *Ihh* and its receptor. Therefore in mice lacking *FGFR3*, *Ihh* expression is upregulated and since *Ihh* stimulates chondrocyte proliferation, this gives an indirect mechanism explaining the reduction in chondrocyte proliferation after activation of *FGFR3* gene [18].

Bone morphogenetic proteins (BMPs) are a group of growth factors, also known as cytokines and as metabologens which were originally discovered by their ability to induce the formation of bone and cartilage. They are now considered to constitute a group of pivotal morphogenetic signals, orchestrating tissue architecture throughout the body [19]. BMP signaling plays an essential role at every stage of endochondral bone development, when injected subcutaneously in mice they induce endochondral bone formation. One of its roles is the formation of mesenchymal condensations. In addition BMP signaling increases the expression of *Ihh* by pre-hypertrophic chondrocytes, thus increases both the proliferation of chondrocytes and the length of proliferating columns of chondrocytes, which oppose the effects of FGF [20].

3.2. Osteoblasts

Osteoblasts arise from stromal mesenchymal cells (MSCs) present in the bone marrow. These cells are multipotent which capable of differentiating into osteoblasts, chondrocytes, adipocytes or myocytes [21]. Osteoblast progenitors were believed to be only bone marrow-derived MSCs. However, other cells were found to be capable of acting as osteoblast progenitors eg. cord blood-borne fibroblast can form complete ossicles, also marrow-residing pericytes can become osteoblasts [22].

Many transcription factors are responsible for MSCs commitment. Among these transcription factors that regulate osteoblast differentiation and required to commit progenitors to pre-osteoblasts are Runx2 (Runt-related transcription factor 2) [23] and Osterix [24]. Wnt/b-catenin Signaling is important in the regulation of osteoblast differentiation [25].

- Runx2, which is also known as core-binding factor subunit alpha-1, is a protein encoded by *RUNX2* gene (6p21.1). Runx2 has a crucial role in the initiation of the osteoblast differentiation process; it is expressed predominantly in cells of the osteoblast lineage and binds to osteoblast-specific cis-acting elements located at the promoter osteoblast-specific gene such as *Col1a1 (17q21.33), osteopontin (4q22.1), osteonectin (5q33), bone sialoprotein (4q22.1),* and *osteocalcin (1q22)* genes [26]. Moreover, Runx2-deficient mice were found to have a cartilaginous skeleton with no true bone matrix formation due to lack of osteoblasts formation [23] and with lacking only one allele of *RUNX2* gene, mice suffer from defects in intramembranous ossification such as hypoplastic clavicles and delayed closure of the fontanelles [23]. This phenotype is closely resembling cleidocranial dysplasia in human, which was subsequently found to be due to heterozygous mutations of the *RUNX2* gene [27].
- Osterix (Osx) is a zinc finger-containing transcription factor that expressed specifically in osteoblasts and its activity is absolutely required in mice for osteoblast differentiation, its inactivation in mice results in perinatal death due to complete absence of bone formation [24]. Mice lacking Osx showed complete lack of osteoblasts and absence of both intramembranous and endochondral bone formation, bones formed by endochondral ossification contained mineralized matrix, but this resembled cartilage, not mineralized bone matrix [24].
  Moreover, Runx2 is essential for *Osx* gene expression as it was found that *Osx* gene is not expressed in Runx2-deficient embryos, but Runx2 is normally expressed in Osx-deficient embryos [24]. Furthermore, the *Osx* gene contains Runx2-binding site in its promoter region, suggesting that Osx might be a direct target of Runx2. These results demonstrated that Osx acts downstream of Runx2 in a transcriptional cascade of osteoblast differentiation [28].
- Wnt proteins (wingless-type MMTV integration site) are a family of secreted glycoproteins (growth factors) that play a critical role in the regulation of osteoblast differentiation and activity in both mice and humans [25]. Activation of the Wnt/b-catenin signaling pathway results in the stabilization and translocation of b-catenin into the nucleus which is an important co-activator that regulates gene transcription [29]. Enhanced Wnt/b-catenin signaling enhances osteoblasts differentiation through intramembranous ossification and Runx2 expression but inhibits chondrocyte differentiation and Sox9 expression [30]. Mutations in the *Wnt 1* gene cause osteogenesis imperfecta and an autosomal-dominant form of susceptibility to early onset of osteoporosis [31].

Mature osteoblasts produce a collagen-rich osteoid matrix, which becomes gradually mineralized over time; the delayed mineralization allows time for collagen cross-linking and the development of large, strong fibers. When the osteoid matrix is deposited by osteoblasts, it has two potential forms depending on its collagen orientation and speed of production. During bone development and fracture healing, woven bone is deposited rapidly and contains disordered collagen fibers, while during adult bone remodeling, the more slowly deposited, and mechanically stronger, lamellar bone has collagen fibers oriented in perpendicular planes in adjacent lamellae [32].

Osteoblasts change functionally and morphologically being columnar with abundant endoplasmic reticulum and rapidly synthesizing collagen during the rapid phases of bone formation. In case the bone

Table 1
Mammalian *FGF* and *FGFR* family [65].

A) FGF family

HUGO/MGI Symbol	Alternative Symbol	Name/Comments	Chromosomal location [66]
FGF1/Fgf1	*aFgf*	Acidic Fgf	5q31.3
	Hbgf1	Heparin-binding growth factor 1	
	Ecgr	Endothelial cell growth factor	
FGF2/Fgf2	*bFgf*	Basic Fgf	10q26.13
	Hbgf2	Heparin-binding growth factor 2	
FGF3/Fgf3	*Int-2*	Int-2 oncogene	11q13.3
	V-Int-2	MMTV integration site 2	
FGF4/Fgf4	*Hst1*	Human stomach tumor oncogene	11q13.3
	Hstf1	Heparin secretory transforming protein 1	
	K-Fgf, Kfgf	Kaposi sarcoma Fgf	
FGF5/Fgf5			4q21.21
FGF6/Fgf6	*Hst2*	Hst2 oncogene	12p13.32
FGF7/Fgf7	*Kgf*	Keratinocyte growth factor	15q21.2
FGF8/Fgf8	*Aigf*	Androgen induced growth factor	10q24.32
	Kal 6		
FGF9/Fgf9	*Gaf*	Glia activating factor	13q12.11
	Eks	Elbow knee synostosis	
FGF10/Fgf10	*Kgf-2*	Keratinocyte growth factor 2	5p12
FGF11/Fgf11	*Fhf3*	Fibroblast Growth Factor homologous factor 3	17p13.1
FGF12/Fgf12	*Fhf1*	Fibroblast Growth Factor homologous factor 1	3q28-q29
FGF13/Fgf13	*Fhf2*	Fibroblast Growth Factor homologous factor 2	Xq26.3-q27.1
FGF14/Fgf14	*Fhf4*	Fibroblast Growth Factor homologous factor 4	13q33.1
	Sca 27	spinocerebellar ataxia 27	
Fgf15		Rodent ortholog of vertebrate *Fgf 19*	
FGF16/Fgf16			Xq21.1
FGF17/Fgf17		Called FGF-13 in some older literature	8p21.3
FGF18/Fgf18			5q35.1
FGF19		Human ortholog of rodent *Fgf15*	11q13.3
FGF20/Fgf20			8p22
FGF21/Fgf21			19q13.33
FGF22/Fgf22			19p13.3
FGF23/Fgf23			12p13.32

B) FGFR family

HUGO/MGI Symbol	Alternative Symbol	Name/Comments	Chromosomal location [67]
FGFR1/Fgfr1	*Flg*	Fms-like gene	8p11.23
	Flt 2	Fms-like tyrosine kinase 2	
	Cek	Chicken embryo kinase 1	
	KAL2	Kallman syndrome 2	
	K-sam	KATO-III cell-derived stomach cancer amplified gene	
FGFR2/Fgfr2	*Bek*	Bacterial expressed kinase	10q26.13
	Cek3	Chicken embryo kinase 3	
	Kgfr	KGF receptor	
FGFR3/Fgfr3	*Cek2*	Chicken embryo kinases 2	4p16.3
	Ach	Achondroplasia	
FGFR4/Fgfr4	*Tkf*	Tyrosine kinase related to Fibroblast Growth Factor receptor	5q35.2
FGFRL1/Fgfrl1	*Fgfr5*	Fgf receptor 5	4p16.3

FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; *FGFR*: fibroblast growth factor receptor-like; HUGO: human genome organization; MGI: mouse genome informatics database.

formation rate becomes slower, they become flatter in shape and less metabolically active [33].

The mature osteoblasts can undergo apoptosis or become osteocytes or bone lining cells. Bone-lining cells are flattened cells, lining both endocortical and trabecular bone surfaces [34]. These cells have much less synthetic function, little cytoplasm or endoplasmic reticulum, less cytoplasmic basophilia and alkaline phosphatase activity. But they have gap junctions and may communicate both with one another and with osteocytes found in the bone matrix. Although for long time they were regarded as “resting” or “quiescent” cells, bone-lining cells express receptors for endocrine and paracrine agents as with mature osteoblasts [34]. Studies showed that bone-lining cells can emerge from quiescence to resume their bone-forming ability by contributing to the anabolic effects of PTH [35] and anti-sclerostin treatment [36].

Moreover bone-lining cells can form active osteoclasts that will proliferate and differentiate into mature osteoblasts eg. after osteoblast ablation. In addition bone-lining cells have the ability to enclose the basic multicellular unit activities on the bone surface. Bone-lining cells also express receptor activator of NF-kB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) and may therefore participate in stimulating osteoclastogenesis, which has been suggested to be particularly important in estrogen deficiency [37].

3.3. Osteocyte and mechanosensing

Mature osteocytes are matrix-embedded cells of the osteoblast lineage having long processes radiate in all directions, but the highest density perpendicular to the bone surface, these processes have gap junction allowing direct contact between the osteocyte and the bone surface, other cells in the bone matrix, and marrow spaces [38].

Osteocytes play a major role in phosphate homeostasis as it expresses multiple proteins concerned with phosphate regulation as Phosphate Regulating Neutral Endopeptidase on Chromosome X (PHEX) which is a metalloendoproteinase found on the plasma membrane of dendrites of osteocytes [39], dentin matrix protein (DMP1), matrix extracellular phosphoglycoprotein (Mepe), and sclerostin. Deletion or mutation of DMP1 results in hypophosphatemic rickets [40].

Osteocytes act as the mechanosensory cells. During movement, osteocytes are stimulated by changes in hydrostatic pressure, direct cell strain, fluid flow and electric fields resulting from electrokinetic effects accompanying fluid flow [38]. Also multiple membrane proteins are involved like integrins, connexins and stretch-sensitive ion channels, and maybe through cilia [41]. The flow of canalicular interstitial fluid is the likely stress-derived factor that informs the osteocytes about the level of bone loading. It leads to the release of intracellular calcium followed by the release of nitric oxide, ATP and prostaglandins and the opening of connexin 43 hemichannels enhancing gap junction functions [38].

Mature osteocytes were found to be more responsive to mechanical stress than young osteocytes as Ca^{2+} signaling significantly changed in mature osteocytes [42]. Moreover mechanical loading suppresses sclerostin, an inhibitor of Wnt-stimulated osteoblast differentiation. Sclerostin is important in limiting the amount of bone laid down in modeling [43].

3.4. Osteoclast and bone remodeling

Osteoclasts are giant multinucleated cells that have the capacity to degrade calcified tissues, derived from hematopoietic precursor cells of the myelomonocytic lineage sharing a common precursor with macrophages [44].

Several secreted molecules and signaling proteins control the osteoclastogenesis, the first one documented in mouse mutation called op/op, the gene mutated in op/op mice encodes the M-CSF growth factor. Binding of M-CSF to its receptor (cFMS) present in osteoclast precursors, stimulates their proliferation, differentiation and inhibits their apoptosis [44]. The second molecule is RANKL which is a key factor for osteoclastogenesis. It is expressed by osteoblasts, osteocytes and stromal cells. When it binds to its receptor (RANK) in osteoclast precursors, osteoclast formation is induced. Also it is responsible for fusion, activation of mature osteoclasts to resorb mineralized bone and cell survival [45]. On the other hand, osteoprotegerin (OPG), which is produced by multiple cells including osteoblasts and stromal cells, when it sequesters RANKL, it prevents the RANK/RANKL interaction and inhibits the osteoclastogenesis [45].

Siddiqui and Partridge [46] reviewed the role of cytokines in osteoclast development and reported that interleukins-1 (IL-1), IL-6 and tumor necrosis factor (TNF) stimulate osteoclast development, while IL-4, IL-18, and interferon- γ inhibit osteoclast development.

Post-natal bone remodeling is a dynamic process of balancing osteoclast-mediated bone resorption and osteoblast-mediated bone formation, with the skeletal responses to changes in loading and physiologic needs is met to ensure mineral homeostasis [47]. Remodeling is initiated by stimulation of osteoclast formation, followed by osteoclast-mediated bone resorption which takes few weeks, a reversal period, lag time between the end of resorption and the beginning of formation it takes four or five weeks, and then a long period of bone matrix formation and mineralization mediated by osteoblasts which takes 3–4 months for one packet (Fig. 1). In adults, bone remodeling leads to complete regeneration of the skeleton every 10 years [48].

Once differentiated, the mature osteoclast capacity to resorb bone depends on its ability to synthesize and mobilize a series of electrolytes and degradative enzymes [49]. The osteoclast has a unique cytoskeleton that enables it to polarize on bone and thus degrade the mineralized matrix. Upon activation of the mature osteoclasts, they adhere firmly to the bone surface, using specialized actin-rich podosomes (actin ring) via vitronectin receptors (αVβ3) [50]. Hence, the resorbing osteoclasts form convoluted, villous-like membrane “ruffled borders” through which osteoclasts secrete protons via an electrogenic vacuolar-type H^+ ATPase (V-ATPase) [49] localizes to the ruffled border leads to dissolution of the mineral phase, as well as a release of enzymes such as lysosomal cathepsins, the phosphatase tartrate-resistant acid phosphatase (TRAP), and proteolytic matrix metalloproteinases (MMPs) allow digestion of organic matrix compounds. The sealing mechanism allows to localize the dissolving and degrading of the mineralized bone matrix, while simultaneously protecting neighboring cells from harm. As the basic multicellular unit advances, osteoclasts leave the resorption site and osteoblasts move in to cover the excavated area, secreting osteoid, which later on, will be mineralized into new bone (Fig. 2) [51].

4. Regulators of bone growth

4.1. Local regulators of bone growth

Factors act locally include the above-mentioned cytokines, M-CSF, RANKL, MMPs as well as some prostaglandins, transforming growth factor-β (TGF-β), epidermal growth factor receptor (EGFR) and BMPs.

Transforming Growth Factor-β controls bone remodeling and maintenance. TGF- β has dual effects on osteoblasts; it was found to stimulate expression of early osteoblast differentiation and bone matrix proteins by osteoblasts and at the same time inhibits late osteoblastic differentiation. TGF-β binding to its receptors activates Smad2 and Smad3. Activated Smad3 physically complexes with Runx2 leading to reduction in the transcriptional and osteogenic activity of Runx2 [52].

The epidermal growth factor receptor is a transmembrane glycoprotein. One of the physiological functions of osteoblastic EGFR signaling is maintaining the pool of osteoprogenitors at an undifferentiated stage in bone, which consistent with the animal study that shows decreasing EGFR activity in osteoblast lineage cells leads to decrease the number of osteoprogenitor cell [53]. EGFR signaling also regulates osteoclastogenesis and bone resorption as it decreases osteoblastic expression of OPG and increases osteoblastic expression of monocyte chemoattractant protein 1 [54].

Several MMPs are expressed in bone and cartilage cells during normal bone development. Knock-out mice models and human genetic studies have demonstrated the major importance of MMPs −2, −9, −13, −14, and −16 in skeletal development [55,56].

4.2. Systemic regulators of bone cells

Many systemic hormones, cytokines, and growth factors influence the birth, death, and function of bone cells [57]. The major systemic regulators are the calcium-regulating hormones; PTH, calcitriol, growth hormone (GH), glucocorticoids, thyroid and sex hormones. Other factors, such as insulin-like growth factors (IGFs) have both systemic and local effects [58].

Parathyroid hormone is the most important regulator of calcium homeostasis. It maintains serum calcium concentrations by stimulating bone resorption, increasing renal tubular calcium reabsorption and increasing renal calcitriol production. PTH stimulates bone formation when given intermittently, but inhibits collagen synthesis at high concentrations [59]. It stimulates bone resorption when given (or secreted) continuously, a process mediated by osteoclasts. It also stimulates gene expression in these cells and increases the production of several local factors, including IL-6, IGF-1, an IGF-binding protein (IGF-BP-5) and prostaglandins [60].

Vitamin D improves calcium and phosphorus absorption and mineral deposition. Also, vitamin D has many other health functions such as immune function. The concentration of plasma vitamin D at birth is directly related to the vitamin D status of the mother. Therefore, mother's skin pigmentation, sunlight exposure and vitamin D supplementation are important determinants. Vitamin D deficiency is widely noted (50–75%) in the pregnant females and in the cord blood [61].

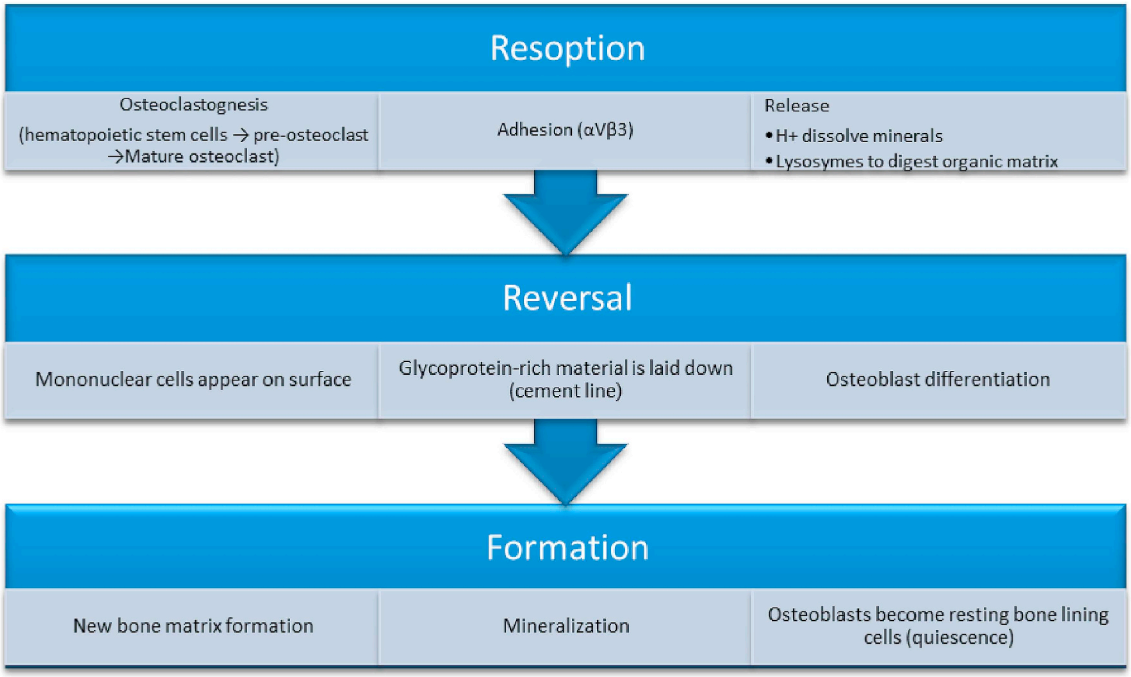
Resoption
Osteoclastognesis
(hematopoietic stem cells → pre-osteoclast →Mature osteoclast)
Adhesion (αVβ3)
Release
• H+ dissolve minerals
• Lysosymes to digest organic matrix
Reversal
Mononuclear cells appear on surface
Glycoprotein-rich material is laid down (cement line)
Osteoblast differentiation
Formation
New bone matrix formation
Mineralization
Osteoblasts become resting bone lining cells (quiescence)

Fig. 1. Schematic representation of the mechanism of bone remodeling.

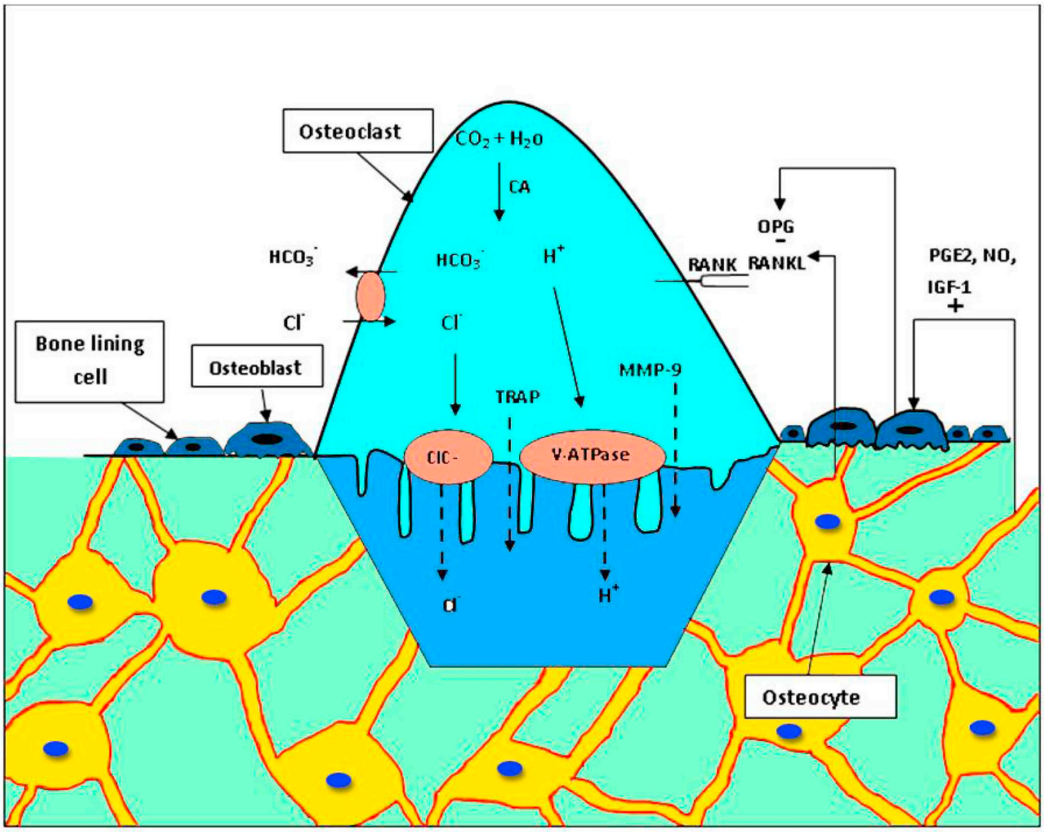

Fig. 2. Schematic representation of cellular interaction in bone remodeling.

Recently, the European Society of Pediatric Gastroenterology and Nutrition Committee on Nutrition of the Preterm Infant (ESPGHAN CoN) recommends a vitamin D intake of 800–1000 IU/day during the first months of life [62].

Growth Hormone has been recognized as the major determinant of circulating IGF-I. Outside the liver, bone is the richest source of IGF-I and IGF-II in the mammalian body. GH has prominent effects on the skeleton in terms of both linear growth and skeletal remodeling. Additionally, IGFs play an important role in bone modeling and acquisition. However, it has been extremely difficult process to ascertain a role for IGF-I independent of GH. The interaction between GH and IGF-I during skeletal growth is complex and has been labeled a “dual-effector” process [63].

Glucocorticoids secreted by the adrenal gland, in physiological doses, are essential for the differentiation and function of osteoblasts and osteoclasts, and they modulate the effects of other hormones and mediators of cell function. They have permissive actions for other hormones in bone allowing them to have their optimal activity. The pharmacological doses of steroids inhibit osteoprogenitor proliferation, osteoblast renewal, osteoblast function, and osteoblast and osteocyte apoptosis. Glucocorticoids also influence osteoclast formation and life span. These direct effects on bone, associated with effects on other systems that indirectly regulate bone metabolism lead to rapid bone loss in patients treated with glucocorticoids [64].

In conclusion, post-natal bone growth is regulated by systemic hormones and locally generated factors. Understanding the dynamic nature of bone tissue, as well as the structural, molecular, and functional physiology of bone cells enables us to obtain a better appreciation of the cellular and molecular basis of bone remodeling and could therefore be valuable in approaches to new therapies.

Funding source

None.

Financial disclosure

The authors have no financial relationships relevant to this article to disclose.

Declaration of competing interest

The authors have no potential conflicts of interest to disclose.

Practical points

- Bone is a dynamic tissue that is constantly being reshaped by osteoblasts and osteoclasts.
- Bone volume is determined by rates of bone formation and bone resorption. The action of osteoblasts and osteoclasts is controlled by several factors that either promote or inhibit the activity of bone remodeling cells.
- Bone remodeling is a process of resorption followed by replacement of bone and occurs throughout life.
- Mechanical strain is one of the most powerful stimulators of bone formation and growth. Therefore, Range-of-motion exercises improve bone mineral content and post-natal bone growth.

Research directions

- Biomarkers of bone turnover can be measured in blood or urine and are used in selective combinations of formation and resorption markers that express the metabolic activity of osteoblasts or osteoclasts respectively.
- Osteopenia of prematurity is still a topic of the day and needs more research for its prevention and treatment.
- Preterm neonates may benefit from range-of-motion exercises in terms of bone mineral content and post-natal bone growth.

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Seminars in Fetal and Neonatal Medicine 25 (2020) 101068

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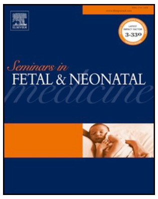

Risk factors of bone mineral metabolic disorders

Alicia Montaner Ramón

Neonatology Unit, Hospital Infantil Vall d'Hebron, Passeig de la Vall d'Hebron 119-129, 08035 Barcelona, Spain

ARTICLE INFO

Keywords:
Metabolic bone disease
Osteopenia
Vitamin D
Prematurity
Neonates
Calcium

ABSTRACT

Bone remodeling is a complex process which integrates different stimuli factors such as mechanical, nutritional and hormonal factors as well as cytokines and growth factors. Bone health depends on an adequate balance between all these factors. The typical bone pathology of the newborn is the metabolic bone disease of prematurity, favored by a lack of mineral accretion in the third trimester of gestation. The intrinsic defects of the bone tissue (primary osteoporosis) are usually of genetic or idiopathic origin and can affect both the term and the preterm newborn. Other risk factors for osteopenia (secondary osteoporosis) include maternal or gestational factors, nutritional deficits (calcium, phosphorus, vitamin D), endocrinological alterations, use of certain medications antagonistic to bone metabolism, mechanical factors and chronic diseases (renal or hepatic insufficiency, intestinal malabsorption, collagen or metabolic diseases). This review examines the risk factors of developing bone metabolic disorders in neonates.

1. Introduction

The bone is a compact and metabolically active tissue that undergoes continuous regeneration, which lets the skeleton have its regenerative and functional adaptation capacity. Although there have been significant advances in the knowledge of the mechanism of the bone remodeling process, there is still much to learn on many aspects of this complex process, which integrates different stimuli such as cytokines, mechanical, hormonal and growth factors [1]. Among all these factors, an adequate balance between the resorption and deposit of phosphorus and calcium in the bone, are essential for a correct bone remodeling [2].

Maintenance of physiologically normal serum calcium and phosphorus requires complex interactions between the kidneys, gastrointestinal tract, and bone. Calciotropic hormones such as vitamin D and parathyroid hormone (PTH), as well as hormones controlling phosphorus homeostasis, such as fibroblast growth factor-23 (FGF-23), are essential in controlling these interactions [2,3].

Many factors regulate bone mass such as nutrition, lifestyle, physical activity and mobilization, hormones, local growth factors and cytokines, genetic factors, etc. Some of these factors can influence bone mass already during fetal and neonatal period, while others will affect it later in childhood, adolescence and adult life [4].

Defects in bone mineralization in the neonate may be due to an intrinsic defect of the bone tissue (primary osteoporosis) or may be caused by different risk factors (secondary osteoporosis or osteopenia). As there are so many factors that influence the development of bone mass, there are also many risk factors for bone mineral metabolic disorders, some of which may already influence during fetal life [4]. The main risk factors of bone mineral metabolic disorders are summarized in Table 1.

Most of the factors that can affect bone development in fetal and neonatal period may be common at term and preterm newborns. However, due to the progressive increase in the survival of extreme preterm infants and the growing interest in the study of pathologies resulting from prematurity, we will make a separate mention for metabolic bone disease (MBD) or osteopenia of prematurity.

2. Metabolic bone disease or osteopenia of prematurity

MBD is a complication of prematurity, characterized by a reduction of osteoid tissue and bone mineral component, and by biochemical alterations of phospho-calcium metabolism [5]. The period of greatest development of the skeleton during fetal life is mainly at the end of the third trimester. The neonate born prematurely is deprived of these necessary contributions for adequate mineralization [6]. This pathology usually develops after the fourth week of life, with wide clinical variability, from mild asymptomatic forms to classic rickets, which favors the production of multiple fractures and alterations of phosphorus-calcium metabolism [6,7].

MBD of prematurity can be caused by several nutritional and biomechanical factors. The most important factor remains the deficit of nutrients (vitamin D, calcium, phosphorus and magnesium), although the immobilization of these children, prolonged parenteral nutrition

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E-mail address: amontaner@vhebron.net.

https://doi.org/10.1016/j.siny.2019.101068

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Abbreviations

PTH	Parathyroid hormone
MBD	Metabolic bone disease
BMC	Bone mineral content

(especially more than 3 weeks) and the use of medications that are opposite or antagonistic to the bone metabolism can also contribute significantly [8].

- **Vitamin D deficiency**: The serum levels of 25 (OH) vitamin D3 in the newborn are related to maternal levels and its insufficiency has been related to an increased risk of osteopenia, respiratory infections in the first year of life, less neurological development to 14 months of age and lower bone mineral mass at 9 years of age. Some studies have described that sufficient or optimal levels of vitamin D protect the premature newborn from the development of MBD, even in neonates with multiple risk factors [8,9].
- **Insufficient calcium and phosphorus intake**: The period of greatest development of the skeleton is during fetal life, mainly at the end of the third trimester. Bone mineralization includes the synthesis of the organic matrix by the osteoblasts, on which the calcium and phosphorus salts are deposited. This process increases exponentially between the 24th and 37th weeks of gestation, reaching 80% of the accretion mineral in the third trimester. The placenta plays an essential role in this period as an active transport of calcium with a 1:4 gradient in favor of the fetus occurs 8. In addition, the placenta is able to hydroxylate vitamin D to its active form, 1,25 (OH) vitamin D3, which is essential to promote the transport of calcium and phosphate to the fetus. The neonate born prematurely is deprived of the contribution of calcium and phosphorus necessary for bone mineralization. The chronic affectation of the placenta, alters the phosphate transport, which can condition a greater osteopenia of children with growth restriction. Demineralization has also been observed in children with maternal chorioamnionitis [10].
- **Prolonged parenteral nutrition**: The contribution of calcium and phosphorus in parenteral nutrition is conditioned by its solubility, so that a dose similar to that reached intrauterine can not be achieved [11].
  In addition, the content of calcium and phosphorus in the preterm breast milk is insufficient in relation to the calculated needs, assuming an absorption of approximately one third of what they would receive in fetal life [8].
  Formula milk is richer in calcium and phosphorus but its bioavailability is lower. Taking breast milk with fortifiers is very important both to prevent and to treat MBD [8].
- **Immobilization**: The fetus in the uterus presents activity and spontaneous movements that decrease in extrauterine life while the newborn remains in the incubator. The reduced muscle activity inhibits the accretion of new bone tissue, favoring bone resorption by osteoclasts and loss of calcium by the kidney. Therefore, the passive physiotherapy performed in these children is important for bone health, provided that contribution of calcium and phosphorus is sufficient [10,12,13].
- **Drugs**: some medications frequently used in the premature newborn such as corticosteroids, methylxanthines, loop diuretics such as furosemide, antifungals and some antiepileptics favor the appearance of MBD. This is due to the fact that they favor the activation of osteoclasts and decrease the proliferation of osteoblasts, and that they decrease absorption and favor the renal elimination of calcium [14,15].

Table 1
Risk factors for bone mineral metabolic disorders in the newborn and early childhood.

Risk factors of osteopenia/osteoporosis
1) *MATERNAL OR GESTATIONAL FACTORS*
- Birthweights of both parents
- Mother smoker
- Intense physical exercise during late pregnancy
- Malnutrition or maternal hypocalcemia
2) *NUTRITIONAL FACTORS/DISORDERS OF CALCIUM AND PHOSPHORUS HOMEOSTASIS*
- Intestinal malabsorption
- Insufficient calcium, phosphorus, magnesium or vitamin D intake
- Chronic renal insufficiency
3) *DEFECTS IN THE SYNTHESIS OF VITAMIN D*
- Insufficient vitamin D intake
- Chronic liver diseases
- Chronic renal insufficiency
- Congenital defects of vitamin D synthesis and receptor
4) *ENDOCRINOLOGICAL FACTORS*
- Thyroid disease
- Hyperparathyroidism
5) *DRUGS*
- Glucocorticoids
- Diuretics
- Calcium antagonists
- Anticoagulants
- Anticonvulsant drugs
6) *MECHANICAL FACTORS*
- Prolonged immobilization
7) *COLLAGEN DEFECTS AND OTHER METABOLIC ALTERATIONS*
- Ehlers-Danlos syndrome
- Marfan syndrome
- Loeys-Dietz syndrome
- Wilson's disease
- Menkes disease
- Homocystinuria
- Hemochromatosis
- Etc.

3. Primary osteoporosis

The intrinsic defects of the bone tissue are usually of genetic or idiopathic origin. The most frequent alterations are produced by mutations in the genes that encode the collagen protein or proteins that are responsible for collagen assembly, although other pathologies that affect other genes, such as alkaline phosphatase, are also described.

The following pathologies affect the bone material already in the fetal or neonatal period:

- **Osteogenesis imperfecta**: Heterogeneous group of genetic disorders with a prevalence of 6–7/100000 newborn [16], characterized by an increase in bone fragility, low bone mass and susceptibility to bone fractures. 95% of mutations are found in the COL1A1 and COL1A2 genes, which code for the alpha 1 and alpha 2 chains of type 1 collagen [7].
  - Type I - Dominant with blue sclerotics, mild form, with normal or slightly low stature and without deformities.
  - Type II - Lethal perinatal, severe fractures at birth (multiple rib and long bone fractures), with significant deformities.
  - Type III - Non-lethal neonatal, severe form with high risk of fractures, very low stature and severe scoliosis, low ears, grayish sclera.
  - Type IV - Dominant with white sclerotics and late presentation. Moderately low stature, mild to moderate scoliosis [7].
- **Bruck syndrome**: Combination of congenital arthrogryphosis and bone fragility/osteogenesis imperfecta with presence of multiple contractures, pterygium and equinovar feet in patients with normal mental state [17,18].
  There are two types of Bruck syndrome, due to mutations in genes than encode collagen binding proteins with autosomal recessive inheritance:

  - Bruck I due to mutation of FKBP10 gene located in the 17p12 chromosome
  - Bruck II due to PLOD2 gene located in the 3q24 human chromosome.
- **Hypophosphatasia**: congenital disease, characterized by bone and dental mineralization defect, secondary to a deficiency in the biosynthesis of the non-specific tissue isoenzyme of bone, liver and kidney alkaline phosphatase. Hypophosphatasia is caused by mutations of the ALPL gene (1p36.12), which encodes the non-specific alkaline phosphatase tissue isoenzyme (TNSALP). It has variable clinical expression, from intrauterine death due to severe alteration of bone mineralization, to early loss of the dentition in adulthood exclusively [7,19].
- **Cole-Carpenter syndrome**: a rare autosomal disease characterized by features of osteogenesis imperfecta (bone fragility and deformities and blue sclera) along with growth retardation, craniosynostosis, hydrocephalus and distinctive facial dysmorphism (ocular proptosis, downslanting palpebral fissures, frontal bossing, hypertelorism, micrognathia, depressed nasal bridge) [20–22].
  There are 3 types of this syndrome:
  - Type 1 caused by mutations of P4HB gene located in the 17q25 chromosome.
  - Type 2 caused by mutations of SEC24D protein transport SEC24D located in the 4q26 chromosome.
  - Type 3 caused by mutations of the gene encoded the cartilage associated protein CRTAP located in the 3p22.3 chromosome of human genome.

4. Secondary osteoporosis or osteopenias

4.1. Maternal or gestational factors

Various maternal, paternal or gestational conditions may affect the bone mineral content of the newborn. Birthweights of both parents and the height of the father have been related to a greater bone mineral content (BMC) in the newborn. These associations were independent of the size of the placenta, a marker of the placental capacity to deliver nutrients [23].

Maternal birth weight is related to neonatal BMC. Various studies that have investigated this relationship suggest that this association is more dependent on the intrauterine environment than on a genotype shared between the mother and the fetus [23,24]. Paternal height and birth weight are independent determinants of neonatal bone mass, indicating a genetic influence on the growth of the fetal skeleton. The constitutive expression of the type 2 insulin-like growth factor (IGF-2) gene with parental imprinting is important for the development of the fetal skeleton [23,25,26].

Some studies have shown that bone mass in infancy and infant growth rates could be strongly influenced by the trajectory of intrauterine growth. Influences in the fetal phase of growth, especially in the last trimester, when the fetal growth rate is higher, could have implications on the risk of osteoporosis.

Maternal smoking has been related to lower birth weight and length. In 2001, Godfrey et al. also demonstrated an effect of maternal smoking on reduced BMC. This could be explained by an impaired placental function, reduced placental blood flow and effects on fetal oxygen carrying capacity. In addition, the cadmium present in tobacco can have a toxic effect on fetal skeletal growth [23,27].

Maternal thinness and intense physical activity during the latter part of pregnancy have also been associated with reduced BMC. More studies are needed on this area, but it seems that it could be related to a lower fetal availability of calcium and/or phosphorus that would cause a lower bone accretion [23].

Other maternal conditions like diabetes mellitus, hypertension, renal failure, preeclampsia, severe malnutrition and extremely low vitamin D status have a deleterious effect on fetal skeletal health. Maternal vitamin D deficiency seems to have a minimal effect on the growth of fetal bone, however, the state of maternal phospho-calcium metabolism does affect the development of the fetal skeleton [10].

4.2. Nutritional factors and disorders of calcium and phosphorus homeostasis

The disorders of calcium and phosphorus homeostasis present acute and chronic clinical consequences for newborns. The balance of calcium and phosphorus must necessarily be positive in order to achieve adequate growth and maturation 3. Any pathology that can influence the balance between the inputs and the elimination of calcium and phosphorus, can affect the growth and development of the growing skeleton 3. The calcium and phosphorus inputs depend on the feeding of the newborn, so the digestive pathologies that alter the absorption of these nutrients can cause problems in the bone metabolism.

Neonates with chronic malabsorption, either due to medical (for example, cystic fibrosis) or surgical (short gut syndrome secondary to intestinal resection due to gastroschisis, necrotising enterocolitis, etc.) are at risk of insufficient calcium, phosphorus, magnesium or vitamin D

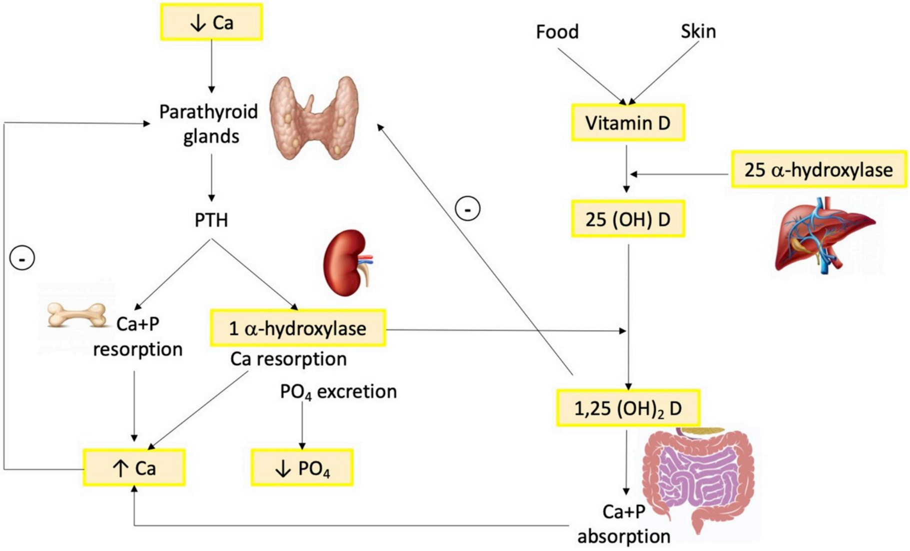

Fig. 1. Metabolism of calcium and phosphorus and its hormonal regulation.

absorption [10].

The renal tubular handling of these minerals is a key control point in the regulation of this balance. The adaptive changes in the renal reabsorption of calcium and phosphorus in newborns explain how a total positive body balance of these minerals is achieved. Renal disorders, like chronic renal insufficiency, that alter this balance, also can affect the growth and development of the growing skeleton [3].

Neonates with conditions that affect their correct nutrition, are also at risk of insufficient calcium, phosphorus, magnesium or vitamin D intake [10].

4.3. Defects in the synthesis of vitamin D

Vitamin D is essential in the maintenance of an adequate bone metabolism (Fig. 1). The main action of vitamin D is to increase intestinal absorption of calcium and phosphorus through its active metabolite 1,25 dihydroxyvitamin D3, in addition to facilitating the maintenance of neuromuscular function and bone remodeling [28,29].

Disorders in which there is a deficit of this active metabolite, could have an increased risk of presenting bone disorders. As an individual has low vitamin D levels, intestinal calcium and phosphorous absorption decrease; serum ionized calcium levels drop, and synthesis of parathyroid hormone (PTH) is stimulated. Increased plasma PTH maintains the serum calcium in normal range by enhancing renal production of 1,25(OH)2D, increasing bone turnover and accelerating bone loss [30].

The most frequent cause of vitamin D deficiency is insufficient vitamin D intake or secondary to a maternal deficit of vitamin D. Other situations that induce a vitamin D deficiency are renal failure, hepatic diseases or congenital defects of vitamin D synthesis and receptor.

In renal disease, the decrease in renal functional mass and the decrease in the synthesis of the active metabolite of vitamin D produce a situation of hypocalcemia that stimulates the synthesis of PTH, favoring bone demineralization [31].

Bone disorders are common in children with liver diseases, especially those in end-stage and those associated with cholestasis. Alteration of hepatocyte function, the malabsorption of liposoluble vitamins and precursor of vitamin D hydroxylation failure are responsible for the bone pathology [32].

Finally, there are two rare genetic diseases that affect the synthesis or the receptor of vitamin D and that can also cause rickets in children. The vitamin D dependent rickets type 1 (VDDR-1) or pseudovitamin D deficiency rickets (PDDR) is produced by a mutation in the gene encoding the 1α-hydroxylase enzyme (CYP27B1 gene) [33]. Children with 1α-hydroxylase deficiency present with a clinical picture of joint pain and deformity, hypotonia, muscle weakness, growth failure and sometimes hypocalcemic seizures or fractures in early infancy. The hereditary vitamin D resistant rickets (HVDRR) also known as vitamin D dependent rickets type II (VDDR II) is produced by a mutation in the gene that encodes the vitamin D receptor. These neonates also present hypocalcemia, secondary hyperparathyroidism and early onset severe rickets.

The clinical finding that distinguishes HVDRR from 1α-hydroxylase deficiency is the serum 1.25 (OH) 2D values, which are low or absent in 1α-hydroxylase deficiency and elevated in HVDRR [33].

4.4. Endocrinological factors

It is well know that endocrinological disorders such as hyperparathyroidism or thyroid disease, can affect bone metabolism. Thyroid hormones are essential for skeletal development and are important regulators of bone maintenance. Hypothyroidism in childhood is relatively common and causes delayed skeletal development, growth retardation and short stature with impaired bone maturation due to defective endochondral ossification. In severe and untreated cases, there is complete post-natal growth arrest [34,35]. In contrast, situations of hyperthyroidism or thyrotoxicosis, which in children are very rare, cause accelerated intramembranous and endochondral ossification and an increase in linear growth rate [36]. In neonates, early closure of the cranial sutures can result in craniosynostosis with neurological sequelae [37]. The opposite skeletal consequences of both situations and the rapid response to treatment, demonstrate that normal euthyroid status during growth is essential to establish peak bone mass and strength in childhood and early adulthood [34].

Hyperparathyroidism also affects bone metabolism producing severe hypercalcemia, bone demineralization and growth and development delay.

Most situations of hyperparathyroidism in the neonate are secondary to insufficient intake of phosphate, calcium or vitamin D [38]. Patients with chronic renal failure may also have elevated PTH, as compensation for hyperphosphatemia. Due to these factors, loss of normal renal function ultimately leads to derangement in mineral and bone metabolism resulting in severe skeletal deformities [39].

Another rare cause of hyperparathyroidism in the neonatal period is Neonatal severe primary hyperparathyroidism (NSPH), a life-threatening condition that presents within days of life with marked hypercalcemia, relative hypocalciuria, and serum parathyroid hormone (PTH) concentrations as much as 10 -fold higher than normal. This pathology is caused by mutations with loss of function of the calcium-sensing receptor (CaSR) gene, which is located on chromosome 3q137, [40,41].

4.5. Medications

The use of certain medications causes bone loss in neonates without other associated risk factors. Many drugs can affect bone metabolism causing bone loss, but in this chapter we will describe the most frequently used in the neonatal population [14].

The adverse effects of **glucocorticoids** on bone metabolism have been known for many years. The most important biological alterations observed by glucocorticoids are a decrease in osteoblastic activity, producing a suppression of bone formation, as well as an induction to osteocytic apoptosis and a decrease in the half-life of osteoblasts. It also seems to be an increase in bone resorption, increasing the half-life of the osteoclasts. Corticosteroids induce a hyperparathyroidism state, either by direct effect on the secretion of PTH, or secondary, induced by the decrease in intestinal calcium absorption and increased urinary elimination. Glucocorticoids can be used frequently in the newborn, especially in relation to respiratory pathology, although normally they are not used for prolonged periods of time [14,42].

The effect of **diuretics** on bone mass seems to be based on its indirect action on calcium balance. Thiazide diuretics stimulate calcium reabsorption through the distal tube, decreasing urinary calcium excretion. Prolonged treatment with thiazides can increase bone mineral density and decrease the risk of fractures. In contrast, loop diuretics, such as furosemide, have an opposite effect, increasing urinary calcium losses [14,43]. When comparing early administration in the first weeks of life with later onset of diuretic treatment, no statistically significant differences were found in the incidence of MBD [44].

The **calcium antagonists** drugs inhibit the absorption of calcium in the mucosa of the small intestine, which can induce a situation of hypocalcemia and secondary hyperparathyroidism. Some members of this family group, such as amlodipine, are used in the treatment of some forms of high blood pressure in the newborn.

Some **anticoagulants**, such as heparin, induce osteoporosis, although the etiological mechanism is not completely clear and seems multifactorial. Generally, in the newborn it is used for short periods of time and the negative effect on the skeleton is minimal [14,45].

Anticonvulsant drugs induce the hepatic cytochrome P450 systems. This system mediates oxidative reactions and increases the hepatic conversion of steroid hormones, including vitamin D metabolites, to biologically inactive products. For this reason, the effect of anticonvulsants on bone has traditionally been explained as a consequence of alterations in the metabolism of vitamin D. Subsequently, it has also been shown that some anticonvulsants also have direct effects on cells responsible for bone remodeling [14,46,47].

4.6. Mechanical factors

The influence of mechanical factors on remodeling is responsible for the loss of bone mass induced by the lack of physical activity.

In the fetus, the bone mineralization is highly dependent on the amount of force applied to bones in utero. The greatest period of bone loading occurs during the third trimester. As the growing fetus pushes against the uterine wall, bone mineralization is increased to withstand the force. Also in the newborn, bone formation and growth are primarily stimulated by mechanical loading on bones. As bone loading caused by external forces increases, bone formation cells (osteoblasts) are stimulated to accelerate production and increase bone strength. In the absence of bone loading, however, bone formation ceases, and cells of bone resorption (osteoclasts) are stimulated to decrease bone strength [12,48].

Preterm infants, because they were born before or during the third trimester, are deprived of this period of maximum bone mineralization during pregnancy, having a higher risk of defects in bone mineralization than full-term infants [12,13].

Demineralization of the skeleton during the neonatal period in the term newborn, may be the result of immobilization due to other illnesses, neurological, neuromuscular or systemic metabolic diseases [10].

In 1996, Frost proposed the mechanostat/mechanical loading model of postnatal bone formation, which states that the primary factor in the development of bone strength is the load (force) placed on the bone. According to his model, this load placed on the bone is transmitted as an input signal to the mechanostat, a sensor inside the bone that is able to assess the strain caused of the load and direct an output to the effector cells, osteoblasts and osteoclasts, based on that strain. Based on the mechanostat model of Frost, a decrease in both fetal and neonatal movements will result in a decrease in bone remodeling [12,49].

Thus, both premature newborns and term newborns can be in risk of defects in bone mineralization due to the state of immobilization and lack of motor stimuli to which they are exposed during time of hospitalization compared to a healthy newborn stimulated at home [12,48–50].

4.7. Collagen defects and other metabolic alterations

Some collagen disorders, although they do not directly affect bone collagen, may be associated with bone disorders. Also some metabolic disorders can affect bone metabolism in the short or long term. The description of these disorders is beyond the scope of this chapter.

PRACTICE POINTS

- Bone mineral metabolic disorders may be favored by genetic, prenatal and postnatal factors.
- The typical picture of bone disorder in the premature newborn is metabolic bone disease.
- Intrinsic defects of the bone tissue (primary osteoporosis) can affect both the term and the preterm newborn.
- Numerous factors (gestational, nutritional, endocrinological, mechanical, medications) and some diseases can induce secondary osteopenia.

RESEARCH DIRECTIONS

- The role of prenatal diagnostic in bone diseases of genetic origin.
- Measures to improve the prevention of bone metabolic disease of prematurity.

Declaration of competing interest

None.

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Seminars in Fetal and Neonatal Medicine 25 (2020) 101086

Contents lists available at ScienceDirect

Seminars in Fetal and Neonatal Medicine

journal homepage: www.elsevier.com/locate/siny

Screening for Metabolic Bone Disease of prematurity

Arpana Rayannavar[a,*], Andrew C. Calabria[b]

[a] *Division of Endocrinology, Department of Pediatrics, University of Minnesota, Minneapolis, MN, USA*
[b] *Division of Endocrinology and Diabetes Children's Hospital of Philadelphia, Associate Professor of Clinical Pediatrics, Perelman School of Medicine at the University of Pennsylvania, 3500 Civic Center Blvd, Buerger Center, 12th floor, Philadelphia, PA, USA*

ARTICLE INFO

Keywords:
Metabolic bone disease (MBD) of prematurity
Calcium (Ca)
Phosphorus (Phos)
Alkaline phosphatase (ALP)
Tubular reabsorption of phosphorus (TRP)
Rickets
Osteopenia of prematurity

ABSTRACT

Metabolic bone disease (MBD) of prematurity remains a significant comorbid condition in preterm, low birth weight infants. As the majority of in utero calcium (Ca) and phosphorus (Phos) accretion occurs during the third trimester, many of these children have inadequate mineral stores and are at risk for deficiencies of Ca and Phos. While fortification of formula has allowed for increased mineral delivery to premature infants, intestinal immaturity prevents optimal absorption. This is compounded by immobilization, delayed establishment of enteral feeds, long term parenteral nutrition and medications that may alter mineral levels. Over time, biochemical changes occur and accompany MBD, with poor bone mineralization during this period increasing the risk for complications such as osteopenia, rickets and fractures. Screening is largely based on risk factors, but despite the 2013 AAP Consensus Statement, there remains significant variation in screening practices across institutions. A combination of laboratory and radiologic testing is often used to diagnose and manage MBD of prematurity, but there exists a lack of consensus on which screening tests and thresholds to use. This is in part related to a lack of normative data and clinical trials for preterm infants, and a result, a lack of evidence-based guidelines on the diagnosis and timing of potential treatment. Biochemical markers, such as serum Phos, alkaline phosphatase (ALP) and parathyroid hormone (PTH), have shown some benefit in the diagnosis of MBD in some studies, but have not always been reproducible. Radiographs may identify different degrees of skeletal changes, but these changes may not be detected until later in MBD development. Other modalities, such as DXA and ultrasound, have also been used, but these may be limited by lack of standards in preterm infants or lack of availability in some centers. Further research, more specifically clinical trials, are needed to determine which combination of tests can detect MBD at its earliest, in order to promote early treatment and prevent short- and long-term complications of MBD.

1. Introduction

Metabolic bone disease (MBD) of prematurity is a common problem for preterm, low birth weight, chronically ill neonates. MBD is characterized by reduced bone mineral content and mineralization, and with increased severity, radiologic evidence of rickets can be observed in 10–20% of ELBW neonates [1]. MBD may also be referred to as osteopenia, osteopathy, or rickets of prematurity. Given the lack of consensus on its definition, the exact incidence of MBD is unknown but it has been estimated to affect approximately 23–60% of VLBW (< 1500 g) and ELBW (< 1000 g) infants [2]. Given the significant prevalence of MBD in preterm infants, routine evaluation of bone mineral status is largely driven by risk factors, such as prematurity, low birthweight, prolonged use of parenteral nutrition, lack of mechanical stimulation, and use of certain drugs such as diuretics and corticosteroids. Until the 2013 AAP recommendations, there was no consensus regarding screening for MBD. However, a lack of normative data and clinical trials on this area remains, and despite these recommendations, screening practices for MBD are largely driven by individual center and physician preference. While there are many screening tests available, choosing which ones to use can often be a challenge.

MBD in preterm infants is usually attributable to calcium (Ca) and/or phosphorus (Phos) deficiency, usually as a result of either decreased intake and/or absorption. Since much of the Ca and Phos accretion in utero happens during the third trimester, pre-term infants are often deprived of these elements that are needed for optimal bone health. Therefore, pre-term infants need special fortified formulas or fortified breast milk, as normal formulas will not provide enough Ca or Phos. However, despite these advances in nutrition/fortification, intestinal

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[*] Corresponding author.
E-mail addresses: rayan005@umn.edu (A. Rayannavar), calabriaac@email.chop.edu (A.C. Calabria).

https://doi.org/10.1016/j.siny.2020.101086

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immaturity can be a limiting factor for optimal absorption of Ca and Phos from formulas, making insufficient Ca and/or Phos stores inevitable in many pre-term infants. Additionally, use of medications such as corticosteroids, methylxanthines and diuretics are common and necessary in some patients, which further contribute to the increased risk of MBD [1].

1.1. Postnatal mineral homeostasis

In order to understand the utility of different screening markers, it is important to identify the undergoing biochemical alterations. Placental transport of Ca and Phos from mother to fetus is thought to occur under the control of parathyroid hormone-related peptide (PTHrP) and parathyroid hormone (PTH). Ca and Phos combine to form hydroxyapatite and promote bone mineralization. However, after birth, this preferential shunt stops, and conditions become less favorable for mineralization, especially in preterm infants whose Ca and Phos stores are incompletely developed.

After birth, serum Ca levels drop, which triggers increased PTH activity. PTH regulates Ca homeostasis by binding to specific PTHR1 receptors that are expressed on target cells, most notably in renal tubular cells and osteoblasts, with subsequent activation of adenyl cyclase and production of the secondary messenger cyclic AMP. In the kidney, PTH directly increases renal tubular Ca reabsorption in the distal tubule and decreases tubular reabsorption of Phos (TRP), as characterized by urinary Phos wasting in the proximal tubule. PTH induces expression of the 1-alpha-hydroxylase enzyme, which catalyzes the conversion of 25-hydroxyvitamin D (25(OH)D) to the active metabolite, 1,25-dihydroxyvitamin D (1,25(OH)2D).

Elevated levels of 1,25(OH)2D increase intestinal absorption of Ca, and to a lesser extent, Phos. PTH also participates in the process of osteoclastogenesis by binding to PTHR1 on osteoblasts and inducing the expression of RANKL (receptor activator nuclear factor of kB ligand) in osteoblasts. Increased RANKL induces differentiation and activity of osteoclasts, which stimulate bone resorption, increase bone turnover and trigger the release of Ca and Phos. As renal effects of PTH are the most potent, the net result is hypercalcemia and hypophosphatemia.

Insufficient Phos intake can also directly lead to hypophosphatemia and can be seen in the first weeks of life. If this persists, this can lead to increased bone turnover and elevations in ALP. A primary deficiency in Phos can be accompanied by normal or low PTH, normo- or hypocalcemia, and increased TRP (due to decreased PTH), with decreased Phos wasting. Phos deficiency can also directly activate 1,25(OH)2D, triggering increased intestinal absorption of both Ca and Phos. In response, serum Phos levels increase, as do Ca levels, which can also lead to hypercalcemia and hypercalciuria, with increased risk for nephrocalcinosis.

With insufficient Ca intake, metabolic changes related to secondary hyperparathyroidism occur and may accompany MBD, especially in high risk children (e.g. chronic lung disease on loop diuretics). In this case, hypophosphatemia occurs in setting of low TRP, with increased urinary Phos wasting due to high PTH levels. Serum Ca levels increase due to PTH-directed effects with increased Ca release through increased bone resorption and increases in renal Ca absorption, as well as intestinal Ca absorption that occurs through increased 1,25(OH)2D. As such, even though Ca intake is reduced, serum Ca levels may be normal or even elevated.

1.2. Screening laboratory studies

According to the recent AAP guidelines, screening for MBD in preterm infants should begin at 4–6 weeks after birth, as clinical rickets is uncommon in the first few weeks of life [1]. The various screening tests available include alkaline phosphatase level (ALP), serum Phos level, serum Ca level, PTH, tubular reabsorption of Phos (TRP), 25(OH)D, and radiologic imaging (Table 2).

Many institutions use varying combinations of the above laboratory tests to screen pre-term infants for MBD in time-manner. According to surveys answered by neonatologists from several Level III NICUs in the US, serum ALP was commonly used as an initial screen for MBD along with Phos and Ca levels. PTH level, TRP, and 25(OH)D were usually obtained as follow up biomarkers by abnormal screen [3]. These results come along with the results in 36 level II and III NICUs in the UK, of which all used serum ALP, Ca, and Phos to monitor for MBD weekly or biweekly. Additionally, some NICUs also used urinary markers, such as TRP, and X-ray (XR) imaging to aid the diagnostic process for MBD. The time of screening with the use of imaging remains controversial and therefore the timing varies among practitioners. One unit of the 36 NICUs screened all infants with XRs at six weeks of age, while others screened according to their individual levels of suspicion [4].

1.3. Serum ALP & Phos

ALP is a glycoprotein enzyme that is produced by multiple tissues and was one of the first enzymes explored in human disease. In the 1960s, separation techniques, including gel electrophoresis, showed that ALP consisted of multiple isoenzymes that could be divided by differences in molecular charge. These studies showed that ALP isoenyzmes were present in different tissues, including bone, liver, kidney, intestine and placenta [5]. Later studies by Crofton et al. in 37 pre-term and 21 term neonates two weeks postpartum found that while the bone isoenzyme of ALP was predominant in term infants, pre-term infants also had detectable intestinal ALP, particularly in breastfed infants in which it may contribute up to 10% of total enzyme activity by the third postnatal week [6]. Additional studies have shown that neonatal total ALP is 90% of bone origin and is thought to be a reflection of overall bone turnover [7,8] and is therefore increased during periods of active growth. In children with vitamin D deficiency and resistant rickets, ALP levels are usually also raised. High Ca retention with increased skeletal uptake following intravenous infusions of Ca are also seen in affected children [9].

ALP is expected to physiologically increase in the first few weeks of life before plateauing around 5–6 weeks of age. ALP has been shown to be significantly greater in healthy preterm (< 34 weeks) compared to healthy term neonates, with earlier week of gestation having the greatest impact on bone biochemical markers [10]. Without adequate mineral intake, further increases in ALP can occur and suggest MBD. While ALP levels are expected to increase without adequate mineral intake, studies in pre-term infants have produced varying results regarding its use as a screening tool for MBD in this population [4].

In 1982, Kovar et al. first introduced the use of serum ALP as a biomarker to test for rickets in preterm infants and suggested a cutoff of five times the upper limit of the normal range in adults (> 1650 U/L). This was based on the serum ALP levels of four of 30 infants who had radiologic evidence of rickets [11,12]. In 1982, Koo et al. also reported correlations between decreased skeletal demineralization on X-rays and elevated ALP level, decreased birth weight, decreased gestational age, and decreased enteral feeds. However, this latter study reported much lower ALP levels than the Kovar study, a mean ALP level of 373 ± 143 IU/L at five weeks in the VLBW infants with radiological skeletal demineralization compared to 274 ± 70 IU/L in those VLBW infants without radiological skeletal demineralization [13].

Multiple recent studies have identified an association between elevated serum ALP and MBD/rickets but have settled on different potential screening thresholds. Hung et al. concluded that a 3 week serum ALP level in infants of GA < 34 weeks (mean GA 29.2 weeks, mean BW 1224.1 g) was a good predictor of MBD changes observed in forearm XRs obtained at term. A serum ALP of 700 IU/L had a sensitivity of 73% and specificity of 74%. That specific cut off value also had the highest predictive value and the lowest negative predictive value [14]. Abdallah et al. found a serum ALP level of 500 IU/L to have the highest sensitivity and specificity in detecting bone disease (osteopenia in

Table 1
Studies assessing ALP in determining presence of MBD.

Study	# of subjects	Gestational Age	ALP cutoff to diagnose MBD
Kovar et al., 1982	30 (4 with rickets)	Mean BW 1580 ± 410 g; GA 31 ± 2.5 weeks	> 1650 IU/L in those with radiologic evidence of rickets
Koo et al., 1982	19	BW < 1500 g	373 ± 143 IU/L at 5 weeks in infants with radiological skeletal demineralization
Hung et al., 2011	46	GA < 34 weeks	700 IU/L – 73% sensitivity, 74% sensitivity in predicting MBD changes observed in forarm XRs
Abdallah et al., 2016	120	BW < 1500 g GA < 34 weeks	500 IU/L -highest sensitivity and specificity in detecting bone disease in knee/wrist XR
Backstrom et al., 2000	43	Median BW 1490 g; Median GA 30.3 weeks	900 IU/L in conjunction with Phos < 1.8 mmol/L – 100% sensitivity, 70% specificity in detecting bone disease according to DXA
Mitchell et al., 2009	18 with radiologic rickets	BW < 1000 g	800 IU/L in 17/18 with rickets
Lee et al., 2017	70	BW 812.8 + 141.1 g; Mean GA 25.3 ± 2.1 weeks	650 IU/L at 4th and 5th weeks correlated with lower BMD, 80% sensitivity, 64% specificity
Figueroas-Aloy et al.	336	BW < 1500 g	500 IU/L

knee/wrist XR) in 120 pre-term infants of gestational age < 34 weeks and BW < 1500 g. Levels were checked weekly after a post-natal age of 8 weeks until there was no longer a concern of MBD. Osteopenic infants in this study also showed statistically significant lower gestational age and birth weight [15]. No statistical differences in Ca and Phos were found in these studies between infants with and without osteopenia.

Backstrom et al. also found that ALP was an important marker in predicting MBD but found that the Phos level could further improve the sensitivity and specificity of predicting low bone mineral content. The authors conducted a prospective study including 43 preterm infants (median gestational age 30.3 weeks, median birthweight 1490 g) and found that a combination criteria of a Phos level less than 1.8 mmol/L (5.57 mg/dL) and an ALP greater than 900 IU/L (measured at 3 weeks, 3 months, and 6 months corrected age) yielded a sensitivity of 100% and specificity of 70% in detecting low bone mineral density according to dual energy X-ray absorptiometry (DXA) that was obtained at 3 and 6 months corrected age [8].

However, a few studies have challenged the notion of an elevated ALP level being an indicator of MBD. Faerk et al. found no association between weekly serum ALP and serum Phos levels of preterm infants below 32 weeks gestational age (mean GA 29 weeks, mean BW 1129 g) and bone mineral content at term [16]. Additionally, in a 2009 study, Mitchell et al. found that ALP level inversely correlated with serum Phos level, gestational age, and BW; however, no single value of ALP by itself was related to radiologic findings of rickets on XR, which was found in 18 of 32 infants. However, ALP levels > 800 IU/L were commonly detected in those with radiologic rickets (in 17 of 18 infants with radiologic rickets). The authors concluded that radiologic rickets was more common in infants less than 600 g, and therefore early screening with imaging in addition to screening with ALP should be performed for this high risk population [17].

In 2007, similar to Faerk et al. Lee at al. assessed the predictive ability of weekly ALP levels by analyzing levels in those with low, fair, and good BMD by DXA scan obtained at term and found different results. The authors found that prolonged low Phos levels at the 2nd and 3rd postnatal weeks and prolonged high ALP levels at the 4th and 5th weeks were correlated with lower BMD values at term in extremely low weight infants. Peak ALP values of 650 IU/L were associated with low bone mineral density with 80% sensitivity and 64% specificity [18]. Figueroas-Aloy et al. assessed 336 premature infants (< 1500 g at birth) by obtaining a DXA scan and ALP/Phos/Ca levels. The authors noted that the most optimal ALP cutoff in order to distinguish between children with and without MBD was 500 IU/L, with mild MBD (ALP > 500 IU/L and Phos ≥ 4.5 mg/dL) found in 13.7% (n = 46) and severe MBD (ALP > 500 IU/L and Phos < 4.5 mg/dL) in 3.3% (n = 11). Moreover, BMD reference values were measured in 279 patients, and in this cohort, those with BMD > 10th percentile (0.068 g/cm^2) at discharge had a 90.3% probability of not developing MBD based on their criteria [19].

Despite the variable results noted above and in Table 1, ALP and Phos remain the most commonly used screening tests, as they are readily available and can be monitored frequently. As a result, the 2013 AAP guidelines recommend biweekly monitoring of serum Phos and ALP levels starting at 4–6 weeks. They additionally suggest further evaluation of a Phos value below approximately 4 mg/dL and/or an

Table 2
Screening of MBD.

	Level of Interest	Key Points
ALP	> 800 IU/L or > 500 and trending up	● Inversely proportional to decreased Phos, decreased weight, and decreased GA. ● Can be elevated in liver disease and may consider obtaining bone specific ALP if etiology unclear. ● Can be increased with copper insufficiency ● Can be decreased with zinc deficiency and glucocorticoid exposure.
Phos	< 1.8 mmol/L (5.5 mg/dL)	● Lower levels correlate with MBD ● More likely to require Phos supplementation if < 4 mg/dL
Ca (albumin corrected)	< 8.5 mg/dL or > 10.5 mg/dL	● Usually normal in preterm infants with MBD ● Low levels indicate high losses or decreased intake. ● High levels indicate over-treatment or could be associated with hypophosphatemia.
TRP	> 95% in the setting of low Phos level	● High TRP suggests low urinary Phos wasting (low PTH or low Phos intake) ● Low TRP suggests urinary Phos loss (often from high PTH)
PTH	> 100 pg/dL	
Vitamin D	< 30 ng/ml	● < 20 ng/ml is Vitamin D deficiency
XR	Osteopenia - rachitic changes, fraying and cupping of metaphyses, fractures	● BMC decreased by at least 20% in order for changes to be seen

ALP level of greater than 1000 IU/L with further laboratory testing and/or imaging [1]. Once mineral supplementation or other treatment is started, monitoring and therapeutic goals are individualized to the underlying deficiency. For infants with hypophosphatemia, Phos supplementation should be adjusted to reach a target serum Phos of > 5.5 mg/dL [14].

It is important to note that other trace element levels should be monitored during the time of serial ALP measurements. Zinc is a cofactor of ALP, which is subsequently displaced by copper. Therefore, in cases of copper deficiency, increased levels of ALP are noted, and in cases of zinc deficiency, low ALP levels are noted. Koo et al. also noted that pre-term infants with rickets/fractures have both increased ALP and increased zinc levels. However, the elevated ALP levels in these infants are not caused by the high zinc levels, instead the high zinc levels in this scenario are due to high bone turnover and subsequent release of tissue (bone) zinc [20]. Additionally, glucocorticoid exposure is associated with low ALP levels, as glucocorticoids can inhibit osteoblast-mediated bone formation. In combination with their stimulatory effects on osteoclast-mediated bone resorption, glucocorticoids affect the delicate balance between bone formation and resorption and contribute to increased bone fragility and altered bone remodeling [21].

1.4. Serum Ca-Phos homestasis

As there is a sixfold accretion of Ca and Phos during the third trimester, premature infants are at high risk of developing Ca and Phos abnormalities [22]. Due to the preferential shunt of Ca through active transport across the placenta, fetal plasma total and ionized Ca levels are greater than maternal levels to promote bone mineralization, but this relative hypercalcemia may suppress fetal PTH secretion and $1,25(OH)_2D$ synthesis. While the fetus is also dependent on the maternal supply of 25(OH)D, PTH and $1,25(OH)_2D$ do not readily cross the placenta. Without these protective mechanisms, fetal plasma Ca concentrations decrease after birth and reach a nadir at about 48 h of life, with more significant decreases in infants with significant perinatal stressors (e.g. infant born to diabetic mother, perinatal asphyxia). In term infants, this nadir is accompanied by an increase in PTH and subsequent increase $1,25(OH)_2D$. In the pre-term infant, these rises are much slower, particularly in critically ill, LBW infants, in whom normal values may not reached for several weeks postnatally.

While one may think a low Ca level may aid in the diagnosis of MBD, the sole measurement of serum Ca is generally not a useful screening test for MBD in pre-term infants, as a normal Ca level can be present at the expense of increased bone turnover in the setting of increased Ca and Phos needs [4]. However, evidence of biochemical hypocalcemia can signify decreased intake/absorption or increased urinary losses of Ca in a preterm infant. Conversely, the presence of hypercalcemia can indicate increased bone turnover as a result of hypophosphatemia (likely due to increased levels of 1,25(OH)2D) or excessive Ca intake relative to the patient's needs and can be associated with hypercalciuria. Therefore, measurement of Ca in conjunction with other laboratory values can be helpful in elucidating the patient's needs, but in isolation, has limitations.

2. Serum PTH & TRP levels

A few recent studies also suggest PTH to be an important biomarker in screening MBD. Until recently, it was speculated that preterm infants had PTH levels that were similar to adults. A recent pilot study validated this premise as preterm VLBW infants (n = 134) without severe morbidity had PTH levels (1.6–9.3 pmoL/l; 15.1–87.7 pg/ml) that were similar to normal adult norms (1–7 pmoL/l; 9.4–66 pg/ml) [23].

In one retrospective study including 66 infants, an elevated PTH (> 88 mg/dL) was present in 85% of ELBW infants who had radiographic evidence of rickets. With introduction of Ca carbonate, a significant decrease in PTH was observed over a period of 6 weeks. In a subsequent prospective study of 49 VLBW infants who had MBD, it was found that a 3 week PTH level was a more specific and sensitive marker of radiographic MBD changes at 6 weeks than a 3 week ALP level. A PTH level of > 180 mg/dL or a Phos level of < 4.6 mg/dL was associated with a sensitivity of 100% and specificity of 94% [24].

Rustico et al. used a PTH cut off of 100 mg/dL to define hyperparathyroidism in order to initiate and study the effects of calcitriol as treatment for MBD. Calcitriol was used in order to suppress high PTH levels, which would then reduce urinary Phos wasting and subsequent MBD changes. Calcitriol treatment was associated with significant changes in measured PTH levels. While improved Ca delivery could have helped lower PTH levels in this population, much of the initial decline in PTH in the first 2 weeks of treatment had occurred before there was a notable increase in enteral Ca intake and is more likely associated with initiation of calcitriol [25].

Additionally, a TRP level in addition to a PTH level can be helpful in distinguishing Ca from Phos insufficiency. TRP measures the fraction of filtered Phos that is reabsorbed and is calculated from the ratio of Phos and creatinine in serum and in urine, levels obtained at the same time. The formula used to calculate TRP is $1- [(Urine_{Phos}/Serum_{Phos}) \times (Serum_{Cr}/Urine_{Cr})] \times 100\%$. A normal TRP typically ranges from 85 to 95% but must be interpreted in the context of serum Phos levels. With low serum Phos levels, an elevated TRP suggests nutritional Phos deficiency and need for Phos supplementation to correct hypophosphatemia. Conversely, TRP can be low in the setting of primary renal tubular damage as well as hyperparathyroidism, and PTH can be used to distinguish between these processes. Therefore, if TRP is low and PTH high, this suggests a Ca deficiency and the need for Ca supplementation to suppress elevated PTH levels [21].

Urine Ca/creatinine ratios are typically more helpful as screens for hypercalciuria, likely due to excess Ca intake or increased urinary loss secondary to medications, such as loop diuretics or methylxanthines. A spot urine Ca/creatinine value of 3.8 mmoL/mmol (1.3 mg/mg) represents the 95th percentile and threshold for further evaluation [5].

For infants on Ca or calcitriol, goals of treatment include normalization of PTH levels, serial monitoring for hypercalciuria with urine Ca/creatinine and normalization of serum Phos level by limiting urinary Phos wasting, as evidenced by increases in TRP [14].

2.1. Serum 25(OH)D

While 25(OH)D levels are often the reason for rickets in older children, a 25(OH)D level has not been shown to be a useful screen for MBD in preterm infants. Normal levels have been previously reported in both premature infants with and without rickets. At one institution, in which standard of care was to give 400 international units per day cholecalciferol, the mean 25(OH)D level in 152 preterm infants screened for possible MBD was 57.8 ng/ml ± 2.0 ng/mL and only 5.9% had Vitamin D levels < 20 ng/mL. These suboptimal 25(OH)D levels were interestingly found to be more common in infants born at older gestational age (≥28 weeks), while hypervitaminosis D was more common in preterm infants born at younger gestational age (< 28 weeks). Therefore, 25(OH)D is not believed to be a common contributor to MBD of preterm infants [26]. It does however suggest that there should be more consideration for weight based dosing of vitamin D as opposed to empiric dosing.

However, in cases of maternal Vitamin D deficiency, short gut syndrome, malabsorptive disorders, and anticonvulsant therapies (e.g. phenobarbital) that increase Vitamin D catabolism, lack of Vitamin D can become a more likely contributor of MBD. In these patients, Vitamin D levels should be assessed and closely monitored.

While 1,25(OH)2D is the metabolically active form of vitamin D, 25(OH)D should be the biomarker measured exclusively for vitamin D status. Serum levels of 1,25(OH)2D are not closely associated with overall outcomes of vitamin D exposure and can be normal until profound vitamin D depletion occurs. In addition, the concentration of

25(OH)D is almost 1000-fold of that of 1,25(OH)2D, and the half-life of 25(OH)D is much longer, implying that the concentration is more stable. On the other hand, 1,25(OH)2D is directly influenced by serum concentrations of Ca and Phos, as well as Ca intake, and as a result, serum values of 1,25(OH)2D frequently fluctuate and should only be checked when a defect in renal 1-hydroxylation is suspected.

2.2. Other bone markers

Limited data exist on markers of bone formation, such as bone specific ALP and serum osteocalcin, and bone resorption, such as C-telopeptide and N-telopeptide. Given the lack of normative data in preterm infants, these are generally considered not useful screening markers [1].

Backstrom et al. showed that bone specific ALP would not provide additional benefit when total ALP could be obtained (the area under the ROC curve was 76.4% for total ALP and 65.8% for B-ALP) [8]. Hung et al. also did not find a difference in the sensitivity and specificity of total ALP and bone specific ALP in predicting bone disease in forearm XR [14]. However, in neonates with cholestasis, measurement of bone specific ALP, or ALP isoenzymes, may be useful, as an increased liver fraction of ALP may elevate the total ALP.

Regarding other bone specific markers, Czech-Kowalska et al. conducted a study in which serum osteocalcin and N-telopeptide along with DXA scans were obtained in 184 pre-term infants (GA $<$ 34 weeks) at term and at 3 months corrected age. The authors found no difference in N-telopeptide levels in infants with lower bone mineral content at 3 months corrected age but found higher levels of serum osteocalcin [27]. As serum osteocalcin levels represent osteoblast activity, but also are derived from bone during osteoclast activity, the authors speculate that serum osteocalcin could represent a marker for bone turnover. Given this isolated finding, more studies are need to more fully evaluate such markers in preterm infants.

2.3. Imaging

Radiographs can reveal various degrees of mineral bone disease, but these changes are not often recognized until later in the development of MBD when a significant degree of demineralization has already occurred. Radiographs may detect osteopenia, rachitic changes, and/or fractures. Most commonly, these changes are noted incidentally on long bones visualized on abdominal or chest radiographs performed as part of neonatal care. Radiographic changes indicative of rickets are not seen until bone mineralization is reduced by at least 20% [28]. A long bone film of the wrist or knee should be obtained to confirm the diagnosis of rickets when clinical suspicion arises. The AAP clinical report recommends rechecking radiographs every 5–6 weeks until improved mineralization [1]. If fractures are noted, additional long bone radiographs should be performed to evaluate for occult fractures.

Dual energy X-ray absorptiometry (DXA) scan is the gold standard in determining whole body mineral density in older children and adults. However, the lack of standard normative data for bone mineral density (BMD) in DXA scan for younger children including pre-term infants along with its lack of bedside availability, ionizing radiation, size compared to the small and sick pre-term infants, and the common presence of movement artifact during scans make DXA difficult to use as a frequent screening tool [4,29]. Additionally, DXA measures bone in two dimensions, therefore only giving an estimate of areal bone mineral density. In children, this measure is highly variable due to bone geometry changes with growth. Fat mass is also overestimated in this population, which have led to development of correction equations and corrected reference values for pre-term and term infants [30].

Peripheral quantitative computed tomography has a number of potential advantages over DXA. It is a three-dimensional non-projection technique that quantifies BMD in the spine, proximal femur, forearm and tibia. Unlike DXA, it is able to separate calculations for cortical and trabecular bone compartments, allowing for calculation of not only BMD but also bone geometry [31]. Unfortunately, while used in different research studies to study various chronic diseases in children, to the best of our knowledge, this has not been studied in this age group.

Unlike a DXA scan, quantitative ultrasound, commonly of the tibia, is inexpensive, portable and free of ionizing radiation, and it can provide information on BMD and the structure of the bone by measuring the speed of sound (SOS). SOS increases and bone transmission time decreases with an increase in bone density and strength. In one longitudinal study, SOS was normal at birth in pre-term infants but declined with time, with the greatest fall in pre-term infants born at less than 26 weeks of age. Additionally, SOS was also negatively correlated with peak ALP in pre-term infants [32]. In a systematic review of 29 studies assessing the use of quantitative ultrasound in preterm infants, the authors found a positive correlation between SOS and gestational age, a postnatal decline of SOS (more prominent fall noted in the more severe preterm infants), and a catch up of preterm infants to term infants at 6–12 months of age. Quantitative ultrasound is still primarily a research tool, as there are limited data on its prediction of fracture risk. However, it holds potential as a screening tool for osteopenia and may be useful in assessing treatment efficacy with longitudinal measurements [29]. Reference values have been devised for both term and pre-term infants but are more specific to the manufacturer's device and no standardized values have been developed to date.

2.4. Suggested screening algorithm

While the 2013 AAP Consensus Statement has provided guidance on risk factors for MBD, enteral nutrition, including Ca and vitamin D, and biochemical testing, we would suggest the use of a more comprehensive screening algorithm (Fig. 1) to help in the early identification and treatment of at-risk infants. We recommend initial screening with serum Ca, Phos, and ALP in all premature infants every 1–2 weeks starting at 4–6 weeks of age. In addition to these labs, we recommend X-ray imaging in ELBW (BW $<$ 1000 g) and/or in very premature infants (GA $<$ 34 weeks) as part of the initial screening, given the higher risk of MBD in this population. If ALP is greater than 800 IU/L or greater than 500 IU/L and trending up, especially in the setting of low Phos, we recommend obtaining additional labs including PTH level, urine studies to calculate TRP, and depending on risk factors, 25(OH)D level. Based on the suggestion of Ca and/or Phos deficiency, targeted mineral supplementation can be initiated with monitoring every 1–2 weeks for potential adjustment in dose. For individuals with significant risk factors for MBD who do not show initial signs of Ca and/or Phos deficiency, we recommend rescreening every 2–4 weeks depending on level of suspicion.

3. Conclusions

While the exact incidence of MBD remains unclear, it continues to remain a significant problem for preterm neonates. The early recognition of MBD remains critical to ensure that treatment can be enacted to prevent short- and long-term complications of MBD and is largely based on risk factors. As MBD is most commonly associated with deficiencies in Ca and Phos, screening practices focus on laboratory investigations that are commonly used in clinical care, such as Ca, Phos, and ALP, in addition to additional screens, such as TRP and PTH, which help in further differentiation of these defects of mineral metabolism. Vitamin D screening is generally less helpful, except in at risk infants. Laboratory screening tests, such as markers of bone formation and resorption, remain in the early stages and are not ready for widespread clinical use. Radiographic evaluation is considered a critical part of screening for MBD as well, but other options beyond x-ray imaging are either not widely available or lack normative data. Using the 2013 AAP guidelines as a starting point, more research is needed to increase our understanding of MBD in preterm infants and to improve the screening,

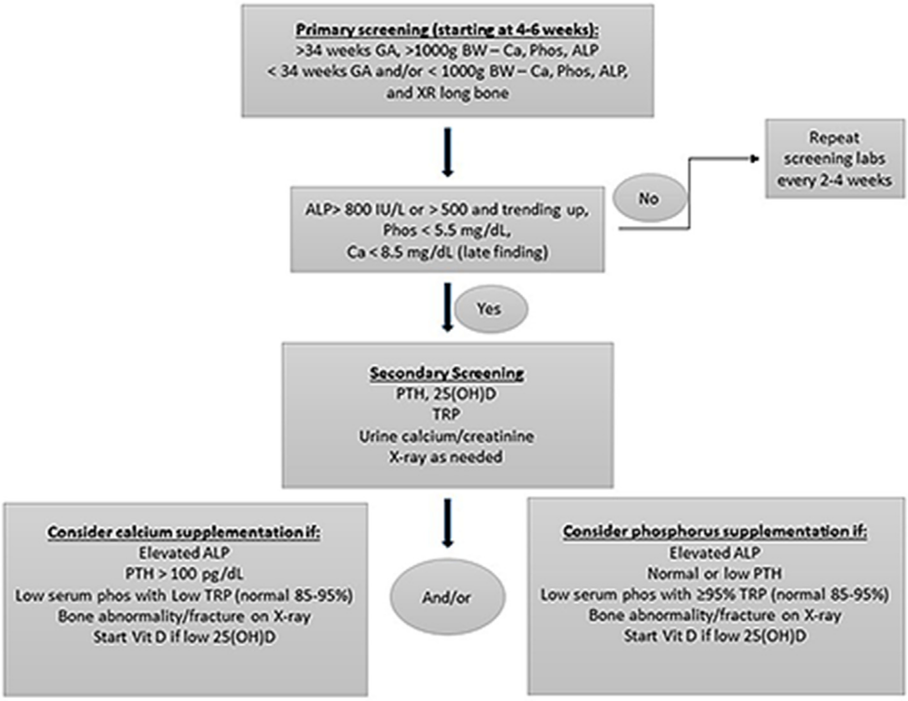
Primary screening (starting at 4-6 weeks):
>34 weeks GA, >1000g BW – Ca, Phos, ALP
< 34 weeks GA and/or < 1000g BW – Ca, Phos, ALP, and XR long bone
Repeat screening labs every 2-4 weeks
No
ALP> 800 IU/L or > 500 and trending up, Phos < 5.5 mg/dL, Ca < 8.5 mg/dL (late finding)
Yes
Secondary Screening
PTH, 25(OH)D
TRP
Urine calcium/creatinine
X-ray as needed
Consider calcium supplementation if:
Elevated ALP
PTH > 100 pg/dL
Low serum phos with Low TRP (normal 85-95%)
Bone abnormality/fracture on X-ray
Start Vit D if low 25(OH)D
And/or
Consider phosphorus supplementation if:
Elevated ALP
Normal or low PTH
Low serum phos with ≥95% TRP (normal 85-95%)
Bone abnormality/fracture on X-ray
Start Vit D if low 25(OH)D

Fig. 1. Screening algorithm for premature infants at risk for metabolic bone disease.

recognition and treatment of this condition.

3.1. Practice points

- Screening for MBD in premature infants is largely driven by risk factors.
- It is most commonly associated with deficiencies in Ca and Phos, as opposed to vitamin D.
- Due to lack of normative data and clinical trials, there is no one established screening practice for MBD in premature infants.
- The most common biochemical screening tools for MBD include serum ALP, Phos, and Ca levels, but each test has limitations. TRP and PTH levels are also important secondary screening tools that can help in diagnosis.
- If laboratory tests are concerning, radiologic X-rays should be considered. However, MBD is not detected on X-ray until more significant mineralization has occurred. DXA has been in some centers but is not widely available. Tibial quantitative ultrasound shows promises but remains primarily a research tool.

4. Research directions

- Improvement in imaging modalities to help in diagnosis of MBD
- Development of consensus guidelines on screening tests and monitoring parameters for children at risk for MBD and potential development of risk scoring system

Declaration of competing interest

No authors have any conflicts of interest to disclose.

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Seminars in Fetal and Neonatal Medicine 25 (2020) 101071

Contents lists available at ScienceDirect

Seminars in Fetal and Neonatal Medicine

journal homepage: www.elsevier.com/locate/siny

Mineral and nutritional requirements of preterm infant

Czech-Kowalska Justyna

The Children's Memorial Health Institute, Department of Neonatology and Neonatal Intensive Care, Al. Dzieci Polskich 20, 04-730, Warsaw, Poland

ARTICLE INFO

Keywords:
Nutrition
Very low birth weight
Osteopenia of prematurity
Vitamin D
25OHD
Calcium
Phosphorus
Extrauterine growth restriction

ABSTRACT

Preterm infants are at risk of growth failure and metabolic bone disease due to insufficient nutrient supply in postnatal life. An ample provision of protein, energy, calcium and phosphates through parenteral or/and enteral nutrition is crucial for bone growth and mineralization. Additional vitamin D supplementation improves bone mineralization and enhance intestinal absorption of minerals.

1. Introduction

The vast majority of preterm infants have low birth weight. The short and long term consequences (including bone health) of immaturity and low birth weight are important from clinical point of view [1]. Very low birth weight (VLBW < 1500 g) infants including those small for their gestational age (SGA) are at a higher risk of reduced bone mineral content (BMC) which might persist up to 6 month of corrected age [2,3]. However, they are able to catch up in length after the postnatal period, and achieve a normal length and bone mineralization at age of nine-ten years [4]. Nevertheless, positive association between birth weight and bone mass up to adulthood was proved in systematic reviews and meta-analyses [1,5].

Preterm delivery disrupts the continuous supply of nutrients through the placenta essential for the normal fetal growth and development. Postnatal growth is mainly determined by genetic and endocrine factors in case of sufficient nutrition, however ensuring adequate amounts of nutrients to premature infants, especially in VLBW infants, is still challenging. Preterm infants have low nutrients stores and higher nutrients requirements than full-term infants. Initially, they are unable to tolerate full enteral feeding because of immaturity of gastrointestinal tract and often need parenteral nutrition limiting at least intake of minerals, protein and calories, and accrual of bone mass [2,3,6]. The goal of nutritional regimens for VLBW infants is to obtain a postnatal resumption of growth at rates observed in utero. Despite of growing improvement of neonatal care still the problem of extrauterine growth restriction exists [7–9]. The incidence of postnatal growth failure in VLBW infants reported at Vermont Oxford Network decreased between 2000 and 2013. However, half of these infants still demonstrated postnatal growth failure [7]. VLBW infants increase their birth weight by 3–4 times and bone mineral content (BMC) by 4–5 times until term corrected age [10,11], therefore they need substantial supply of protein, energy, calcium, phosphate and other nutrients for adequate bone growth and mineralization. The most important factors responsible for further growth improvement in VLBW infants are earlier attainment of first and full oral feedings, greater use of breast milk and restriction of co-morbidities associated with prematurity such as necrotizing enterocolitis [9,12]. The volume of human milk provided during the first 10 days has also positive impact on BMC at term corrected age [13]. The big concern is still the linear growth lagging behind weight gain [8]. Lower linear growth is closely connected to insufficient mineral supply (predominantly phosphorus) and metabolic bone disease of prematurity [13,14]. The nutrients essential for growth are protein and energy. However, suitable intake of protein and energy is also necessary for mineralization of newly formed bone and growth plate cartilage. In the meanwhile vitamin D plays a crucial role in increasing the efficacy of the skeletal mineralization and intestinal absorption of calcium and phosphate.

2. Energy and protein

Energy and proteins are needed for growth and metabolic functions. The recommended energy intake depends on energy expenditures and type of feeding regimen. Higher energy expenditure for digestion and loses of nutrients with stool during enteral feeding should be compensated by higher intakes. Higher energy expenditure is also observed during thermogenesis, rapid growth and physical activity also recognized as an increased work of breathing [15]. Recommended energy

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E-mail address: j.kowalska@ipczd.pl.

https://doi.org/10.1016/j.siny.2019.101071

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intakes are 110–135 kcal/kg/d during enteral nutrition [16], while for parenteral nutrition (PN) is approximately 90–120 kcal/kg/d, except on the first day of life which is characterized by reaching 45–55 kcal/kg/d [17]. Even higher caloric intakes might be essential for infants with increased energy demands e.g. infants with severe chronic lung disease or those with increased losses via intestinal stoma [18].

At the context of bone metabolism, the content of lipid emulsions used in PN as a source of energy might be an issue. Evidence from animal studies suggest that the addition of long-chain polyunsaturated fatty acids (LC-PUFAs), even at suboptimal levels, may have a beneficial effect on bone. However, the role of LC-PUFAs is more complex with opposite effects on osteoclastogenesis and osteoblastogenesis between n-3 and n-6 LC-PUFAs. A reduced dietary n-6:n-3 LC-PUFAs ratio may provide protection against bone mass loss and promote bone formation in early life. The extent of this protective effect on bone mass is dependent on both the amount as well as the specific n-3 PUFAs provided. Trials comparing fat emulsions with high content of arachidonic acid (ARA) and docosahexaenoic acid (DHA) and lower n-6:n-3 ratio (e.g. fish oil, SMOF lipid) with soybean oil emulsion with no ARA, and DHA content, in preterm infants give conflicting results, but the effect on bone was not a primary outcome [19]. Vasudevan et al. [20] reported decreased total serum ALP and increased serum phosphorus in SMOF lipid group compared with soybean group, while Fallon et al. observed an apparent decrease in fracture incidence in infants on prolonged PN (> 4 weeks) neonates receiving 100% fish oil compared with those receiving soybean oil. Furthermore, 67% of the neonates on the soybean oil nutrition had extremity fractures compared with 16.7% of those on the fish oil, who exhibited mostly rib fractures [21].

2.1. Parenteral protein intake

Recently published guidelines [22] decreased maximum recommended dose of parenteral amino-acids in routine practice to 3.5 g/kg/d, because there is limited evidence of beneficial effect of higher amino acids intakes [23]. However, parenteral amino acids should be started with lower dose of at least 1.5 g/kg/d immediately after birth and increased the next day to at least 2.5 g/kg/d. Intakes above this level should be accompanied by an energy intake of > 65 kcal/kg/d. A minimum of 30–40 kcal per 1 g amino acids is usually recommended to guarantee appropriate amino acids utilization [22].

2.2. Enteral protein intake

Enteral protein intake, which is assumed to ensure growth velocity approximating fetal values, is 4.0–4.5 g/kg/d for preterm infants weighting < 1000 g and 3.5–4.0 g/kg/d for infants weighting 1000–1800 g [16]. Enteral protein intakes higher than those administered parenterally appear to be well tolerated and safe in preterm infants [18]. Protein content of human breast milk is not enough to fulfill preterm infants requirements. In order to meet recommended intakes in premature infants the use of a multi-component breast milk fortifier containing both macro- and micronutrients will be required, adding 0.8–1.5 g protein per 100 ml milk depending on the manufacturer [18]. Protein supplementation of human milk increases the rate of growth during hospital stay, but long-term persistence of the positive effect is questionable [24,25]. In some infants additional protein intake from commercial protein supplements might be required [26]. However, precise indications, optimal intakes and when to start and stop human milk fortification, still remain a matter of discussion [18].

3. Calcium and phosphorus

Calcium and phosphorus are the major ions of human skeleton, up to 98% of calcium and 80% of phosphorus is stored in bones with Ca:P molar ratio about 1.7:1. The highest rate of minerals accretion in skeletal system accompanying by the fastest growth of fetus takes place in the third trimester of pregnancy. The peak accretion rate of calcium and phosphorus is 120–150 mg/kg/d and 50–85 mg/kg/d, respectively [27]. The intrauterine accretion rates for calcium and phosphorus has been proposed as a reference intakes for growing preterm infants. Preterm infants miss out the third trimester phase of mineral accretion and therefore they are even more vulnerable to the effects of inadequate mineral provision in the postnatal period. Particularly, an achievement of recommended intake of calcium and phosphorus is challenging, at least during first days of life. It should be underlined that prolonged suboptimal intake of phosphorus increases risk of osteopenia of prematurity. A clinical neonatologist should ensure optimal supply of calcium and phosphorus, irrespective of feeding regimen.

3.1. Parenteral nutrition (PN)

The early use of PN promotes positive nitrogen balance, decreases postnatal weight loss, improves growth and in case of adequate mineral supply ensure bone growth and mineralization. Previously PN does not provide optimal intake of calcium and phosphorus in preterm infants because of solubility issues. Currently available organic compounds of these minerals (sodium glycerophosphate and calcium gluconate) ensure their safe supply in PN, even at the highest recommended doses, without the risk of precipitation and the need of using saturation curves [28]. These high minerals infusions should be given through a central venous line and not through peripheral line.

In consensus [29], the recommended intake of minerals depends on the phase of growth and reads as follow:

Preterm infants during the first days of life:

- Ca: 32-80 mg/kg/d (0,8-2 mmol/kg/d)
- P: 31-62 mg/kg/d (1-2 mmol/kg/d)

Growing premature infants.

- Ca: 64-140 mg/kg/d (1,6-3,5 mmol/kg/d)
- P: 50-108 mg/kg/d (1,6-3,5 mmol/kg/d)

The negative influence of early and high amino acid intake on mineral homeostasis in case of limited phosphorus supply in PN must be stressed [18,30]. High amino acids protein intake without adequate phosphorus supply leads to significant tissue deficit and hypophosphatemia, due to the increased transport of phosphorus (also potassium) into the cells. This condition is named as refeeding-like syndrome in preterm infants. Bonsante et al. [30] showed that the severity of hypophosphatemia accompanied by hypercalcemia and hypercalciuria is proportional to the cumulative deficit of phosphorus and the dose of amino acids. The highest prevalence of hypercalcemia and hypophosphatemia was observed in a high amino acid group. This study highlights the risk of early minerals imbalance while providing “early aggressive” nutrition with postpone phosphorus supply till the 2nd and 3rd day of life.

The reported incidence of early hypophosphatemia was up to 70% in infants of birth weight ≤1250 g during PN without phosphorus supply until the third day of life [31]. Introduction of phosphorus in PN from the first day of life at a dose 0.5–0.8 mmol/kg/d (up to 1.5 mmol/kg/d or more in case of hypophosphatemia) allowed to decrease the prevalence of hypophosphatemia (< 4 mg/dl) to 29.6% on the first week of life. No cases of hypernatremia and hyperphosphatemia were observed [31]. Higher mineral intakes in PN (Ca: 75 vs 45 mg/kg/d; P: 44 vs 26 mg/kg/d) from the first day of life prevents decreasing of bone strength during postnatal period without any side-effects in infants born at < 33 weeks’ gestation [32]. In early PN when minerals intake is relatively low, there is a strong recommendation to use a molar Ca:P ratio below 1.0 (0.8–1.0) to reduce the incidence of early postnatal hypercalcemia and hypophosphatemia. For stable growing infants, in order to achieve the fetal body composition the optimal ratio should be by 1.3 [29]. In hypophosphatemic patients Ca:P ratio < 0.8 allows quickly normalize phosphorus concentration in serum. However, after a few days serum calcium level might drop down and start to stimulate the secretion of PTH which leads to a loss of given phosphorus in urine. Therefore lower than 0.8 ratios might be used only for short period of time and under control of calcium and phosphorus in serum and urine.

The need to begin phosphorus supply from the first days of life in increasing the amounts to the upper recommended intake must be a priority in order to protect against an early hypophosphatemia and to ensure ample phosphorus provision for replenishment the tissue stores and not disturbing bone mineralization. Among preterms, SGA infants and fast growing infants present higher demand for phosphorus in PN because of higher retention [33]. The calculation of phosphorus intake should be related to calcium and amino acids intake and might be based on the following equation: P intake (mmol/kg/d) = [Ca intake (mmol/kg/d)/1,67 + protein intake (g/kg/d) x 0,3].

3.2. Enteral nutrition

Enteral nutrition should be initiated as soon as possible, while simultaneously weaning TPN, but ensuring an adequate nutrients supply for the best growth possible. Current clinical practice promotes early enteral feeding with increasing amounts of preferably own mother milk. Calcium absorption depends on calcium and vitamin D intake, while calcium retention is related to absorbed phosphorus [16]. In preterm infants, intestinal calcium absorption is initially a passive process facilitated by lactose, but later is active and calcitriol dependent [34]. Even preterm infants born < 28 weeks gestation are able to synthetized calcitriol [35]. However, calcitriol's role can be bypassed by increasing the dietary calcium content, or by parenteral administration of calcium [36].

Recommended enteral intake of minerals is higher because intestinal calcium absorption is only around 50%–65% and phosphorus absorption around 80%–90% [16,37]. In human milk, the molar Ca:P ratio is approximately 1.5:1 while mass ratio is by 2:1. The current recommendation for preterm formula Ca:P ratio is similar to that in human milk, however lower mass ratios between 1.5:1 to 2:1 in case of calcium sources with better fractional absorption might be valuable [16]. In Europe, a considerably lower target for calcium an phosphorus intake is proposed than in US (Table 1) [16,38].

The content of calcium (≈20 mg/100 ml) and phosphorus (≈15 mg/100 ml) in breast milk is far too low to meet preterm infants requirements, even at an intake of 150–200 ml/kg/d. Taking into account limited enteral absorption and renal losses, minerals retention constitutes approximately 20–30% of fetal retention from third trimester of pregnancy [16]. Therefore expressed or donor breast milk is commonly supplemented with commercial human milk fortifier (HMF) to meet the additional nutritional needs of preterm infants. A recent meta-analysis showed that multi-nutrient fortification of breast milk increased the rate of growth during hospital stay, but not any longer. Only very limited data are available for bone mineralization. Meta-analysis of data obtained from five trials (restricted to trials without mineral supplementation of the control group) showed that the intervention group had statistically significantly lower serum alkaline phosphatase levels, however clinical significance might be questionable [25]. Einloft et al. study which is not included into the abovementioned meta-analysis, showed improvement in bone mineralization (BMC) in VLBW infants fed human milk with HMF in comparison with infants receiving human milk only. Additionally serum alkaline phosphatase levels were significantly lower (391 ± 177 vs. 720 ± 465 IU/L; $p = 0.007$), what corresponds with a lower risk of osteopenia of prematurity [39].

To maximize nutrient intake increasing feeding volume and/or dose of HMF is recommended [38]. One might also consider an addition of only calcium and phosphorus supplements to human milk to improve bone growth and mineralization in preterm infants, however there is not enough data to support such an attitude especially in the case of wide availability of the multi-component HMF [40]. Additional calcium and phosphorus supplementation might be consider only in case of hypophosphatemia and/or hypocalcemia despite breast milk fortification or formula feeding. The need for direct minerals supplementation might be valuable also in case of essential fluid restriction or inability to tolerate preterm formula or HMF [38]. In general, calcium glubinate or calcium gluconate are most widely used, however supplementation between oral feeding is preferable to avoid diminishing phosphorus absorption. An ideal oral form of phosphorus for enteral use in preterm infants does not exist and there are local specifics.

In case of lack of human milk (own mother or donor milk) the use of increasing amount of preterm formula (PTF) is recommended [38]. The use of PTF is associated with higher rates of weight gain and head growth (although not length gain) during hospital stay [41].

3.3. Post-discharge enteral feeding

Infants with an appropriate weight for postconceptional age at discharge should be fed with expressed breast milk or even breast-fed when possible [42]. However VLBW infants who are discharged exclusively breastfeeding may be at risk of hypophosphatemic rickets and need close calcium and phosphorus homeostasis monitoring [38]. Formula-fed infants may be given term infant formula with LCPUFAs. Infants discharged with a subnormal weight for postconceptional age and thus with an increased risk of long-term growth failure, should be fed human milk with HMF or special post-discharge PTF (in case of lack of breast milk) to provide an adequate nutrient intake, at least until a postconceptional age of 40 weeks, but possibly until about 52 weeks [42]. Preterm infants fed with post-discharge PTF with higher content of minerals and vitamin D have higher BMC increment in comparison to infants on term formula or human milk [43,44]. However, the favorable effects of post-discharge PTF at 6-mo corrected age either were not maintained or could not be confirmed because of high rate of attrition at the age of 8 years [45]. A Cochrane review which included 16 trials compared feeding infants with post-discharge PTF versus term formula did not find consistent evidence of positive effects on growth up to 12–18 months post term [46]. Only five trial assessed influence of post-discharge PTF on bone mineralization, but no significant differences in BMC or bone strength up to 12 month corrected age was found in four trial. However, in clinical practice post-discharge PTF might be an valuable source of minerals for VLBW infants with osteopenia of prematurity.

4. Vitamin D

Vitamin D is a fat-soluble vitamin and the universal biochemical marker of vitamin D status is serum 25-hydroxyvitamin D level (25OHD). There is ongoing debate focused on skeletal or also non-

Table 1
Recommendations for enteral calcium, phosphorus and vitamin D intake, and target 25OHD levels for preterm infants.

	Vitamin D (IU/d)	Target 25OHD level	Calcium (mg/kg/d)	Phosphorus (mg/kg/d)
AAP 2013	200-400 (< 1500 g) 400 (> 1500 g)	> 20 ng/ml (> 50 nmol/l)	150–220	75–140
ESPGHAN 2010	800–1000	> 30 ng/ml (> 75 nmol/l)	120–140	65–90

skeletal health benefits of vitamin D and target 25OHD levels (> 20 ng/ml vs. > 30 ngml) also in preterm infants [16,38,47]. Parathormone (PTH) level is postulated as a functional marker of vitamin D status. Taylor et al. [48] showed a pattern of decreasing in PTH level with improving vitamin D status until reaching a plateau at 25OHD level of 42.9 ng/ml (95% CI 32.2, 53.6) in preterm infants. Additionally, a threshold of 25OHD level above which bone mineralization no longer increased was identified at 48.2 ng/ml (95% CI 35.4, 61) [48]. These values, twice much higher than lower target level recommended by AAP in 2013, might be interpreted as optimal for bone health in preterm infants [38].

4.1. Vitamin D status in preterm infants at birth

The recognized problem of vitamin D deficiency (25OHD level < 20 ng/ml) in preterm infants is worldwide. Vitamin D deficiency is reported in 25–64% of US infants [49,50], 36% of the Australian infants [51], 44% of Brazilian infants [52], 72% Turkish infants [53] (< 32nd gestational week), and also in 72% more mature Greek babies born at 32–36 weeks' gestation after birth [54]. A very high prevalence of vitamin D deficiency is also reported in Indian (86%), Tunisian (74%), Irish (79%) and Czech (92%) VLBW infants [55–58]. The risk of vitamin D deficiency increases with decreasing maturity, thus infants born below 28 weeks have a higher risk (OR = 2.4) than infants born in 28th – 32 nd week [49]. In addition prenatal vitamin D deficiency is at least in part responsible for preterm delivery, increasing this risk by 28% [59]. There is strong evidence that maternal vitamin D supplementation during pregnancy is reasonable solution to overcome the problem of neonatal vitamin D deficiency [60].

Fortunately, vitamin D supplementation after birth substantially improves vitamin D status in preterm infants in a few weeks/months, depending on the prescribed dose, body weight and maturity [48,50,54–57]. Unfortunately, in the last decade a 10-fold increase of rickets incidence was noted, and the majority of children (78.4%) were born preterm. The adjusted odds ratios by birth week were 182 (95% CI: 121–272) before 32nd gestational week and 10.8 (95% CI: 6.72–17.4) by 32nd and 36th gestational week [61]. The substantial risk of vitamin D deficiency and rickets exists also for SGA infants as also in preterm infants with necrotizing enterocolitis due to prolong PN with low vitamin D intake [54,61].

4.2. Effects of vitamin D supplementation in preterm infants

Unfortunately, there is a shortage of clinical trials assessing the effects of vitamin D supplementation in preterm infants, however a few new trials have been published after 2010. Vitamin D supplementation at a dose of 200 IU/d seems that is not sufficient to protect from vitamin D deficiency at hospital discharge preterm infants born ≤32nd gestational week [50,57]. The positive predictors of vitamin D status were a longer duration of supplementation and a lower gestational age at birth. The fastest and more distinct increase in serum 25OHD level (at 40 weeks postmenstrual age) with vitamin D intake at a dose of 800 IU/d rather than 400 IU/d was also observed in RCTs in preterm infants (28–34 gestational week), but not accompanied by better bone mineralization or any side effects [62,63]. Similarly, vitamin D supplementation at a dose 800 IU/d (vs. 200 IU/d, vs. placebo) was most effective in decreasing the baseline high rate (67%) of vitamin D deficiency in infants born < 28th gestational week to 0%, 16% and 41%, respectively [64]. Nevertheless, the majority of infants in 800 IU/d group reached 25OHD level > 60 ng/ml, but there was no clinical and biochemical evidence of toxicity. In population of very high risk of vitamin D deficiency at birth, even higher doses may be required to achieve optimal 25(OH)D levels during hospital stay [56,58,65]. Vitamin D status is more effectively improved during vitamin D supplementation at a dose of 1000 IU/d than 400 IU/d in preterm infants of high risk of vitamin D deficiency at birth [58,65], with lower incidence of skeletal hypomineralization and hyperparathyroidism, and better growth in VLBW infants [58]. Elevated vitamin D levels were seen in 9.8% of infants on 1000 IU/d, but with no clinical or biochemical evidence of toxicity [65]. Vitamin D at a dose 1000 IU/d is more effective than 800 IU/d and 400 IU/d to improve vitamin D status in preterm infants (24–32 gestational week) with high vitamin D deficiency rate (72%). The average 25OHD level at 36 weeks postmenstrual age was significantly higher in 800 IU/d (40 ± 21.4 ng/ml) and 1000 IU/d group (43 ± 18.9 ng/ml) when compared to 400 IU/d group (29.4 ± 13 ng/ml). The prevalence of vitamin D deficiency (2.5% vs 22.5%; RR: 0.09; CI:0.01–0.74) was only significantly lower in 1000 IU/d group when compared to 400 IU/d group [53]. There was no significant difference between 800 IU/d and 400 IU/d group.

4.3. Vitamin D enteral intake

The crucial sources of vitamin D for preterm infants are diet and supplements. Vitamin D intake from diet is low in VLBW infants during the first days of life due to small amount of ingested milk. Preterm formulas are supplemented, but vitamin D content differs widely (50–240 IU/100 ml). The vitamin D content of PTF is approx. 120–190 IU/100 ml in North America, and 70–120 IU/100 ml in Europe, whereas in post-discharge PTF it is approx. 50–70 IU/100 ml. The HMF also ensure different amounts of vitamin D according to the type of fortifier [66]. Therefore vitamin D intake from formula and/or HMF should be always calculated, to protect preterm infants against vitamin D overdosing. A higher risk of vitamin D overdosing is associated not only with higher total intake but also lower body weight [38,67,68]. On the other hand vitamin D concentration in human milk is very low (approx. 5 IU/100 ml) [69], but might be markedly increased by maternal vitamin D intake at a dose of 6000 IU/d [70]. This action improves infants' vitamin D status even in high risk populations but lower intake < 2000 IU/d has no significant effect [71].

The biggest concern is a lack of international consensus on optimal vitamin D intake and target 25OHD levels for preterm infants. The recommended vitamin D intake for preterm infants varies from 200 IU/d up to 1000 IU/d. The American and European recommendations based on the limited data and different approach to target 25OHD level are presented in Table 1, [16,38]. Undoubtedly, to obtain 25OHD level > 30 ng/ml in preterm infants with very high risk of vitamin D deficiency at birth, higher vitamin D intake is necessary [47,72]. The higher (800 IU/d) initial dose will help to quickly ensure optimal vitamin D status [64,72]. However, their smaller size may lead to a lower need for vitamin D to keep a satisfactory 25OHD level [38,72]. When achieving a total dose of 1000 IU/d, combining supplements and diet, there is a risk of vitamin D overdose, particularly in neonates with birth weight < 1000 g [47,72]. Based on the results of abovementioned intervention trials and recently published national guidelines [47], I would recommend:

- For preterm neonates born below 33 gestational week: to start supplementation at a dose of 800 IU/d from the first days of life under the control of 25OHD level (a first control after 4 weeks of supplementation)
- For preterm neonates born at 33–36 gestational week: 400 IU/d from the first days of life, regardless the way of feeding; supplementation under the control of 25OHD level should be considered in infants from the risk groups (PN > 2 weeks, ketoconazole > 2 weeks, anticonvulsant treatment, cholestasis and birth weight < 1000 g)

5. Conclusions

Preterm infants have low nutrients stores and higher nutrients requirements than full-term infants. For bone growth and mineralization substantial intake of protein, energy, calcium, phosphorus and vitamin

D is necessary. Enteral nutrition should be initiated as soon as possible when the infant's medical condition allows, while simultaneously weaning TPN, at the same time ensuring provision of adequate nutrients for the best growth and bone mineralization possible. In case of nutrition based on human milk multi-nutrient fortification is reasonable, at least during hospital stay. Vitamin D supplementation is also essential.

A research agenda

Further trials are needed to determine the exact target 25OHD level and adequate vitamin D dose for preterm infants (especially for VLBW infants), considering skeletal and non-skeletal effects.

Practice points

- Preterm infants are at risk of extrauterine growth failure and metabolic bone disease due to insufficient nutrient supply in postnatal life.
- Phosphorus should be introduced to PN from the first days of life to protect against refeeding-like syndrome.
- To ensure appropriate protein, energy, calcium and phosphorus intake human milk with HMF or preterm formula (in case of lack of breast or donor milk) are recommended for preterm infants.
- Vitamin D deficiency is very common in preterm infants at birth
- Supplements are the most important source of vitamin D, however vitamin D intake from diet should be also calculated, because prolonged vitamin D intake at a dose > 1000 IU/d increases risk of vitamin D overdosing at least in infants born below 28 gestational week.

Founding source

None.

Declaration of competing interest

Non declared.

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Seminars in Fetal and Neonatal Medicine 25 (2020) 101075

Contents lists available at ScienceDirect

Seminars in Fetal and Neonatal Medicine

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Current status in therapeutic interventions of neonatal bone mineral metabolic disorders

Amish Chinoy[a,b], M. Zulf Mughal[a,b], Raja Padidela[a,b,*]

[a] Department of Paediatric Endocrinology, Royal Manchester Children's Hospital, Manchester, UK
[b] Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK

ABSTRACT

Neonatal care has significantly improved in the past decade with improved survival of preterm and sick neonates. Similarly, the field of bone and mineral disorders is continuing to accelerate with better understanding of pathophysiology and genetic basis of diseases, as well as availability of newer diagnostic and therapeutic modalities. In this extensive and rapidly expanding field, metabolic bone disease specialists are frequently called upon to translate progress into better care for neonates with bone and mineral disorders. Accordingly, this chapter provides a review of clinical manifestations and evidence-based investigation and management (where available) of common, rare and ultra-rare disorders of bone and mineral metabolism manifesting in the neonatal period. Besides medical treatment we emphasise the crucial role of the multidisciplinary team, which include physical therapists, occupational therapists and dieticians, in the care of neonates with bone disorders such as osteogenesis imperfecta and achondroplasia.

1. Introduction

Bone is a composite structure made up of organic (collagen and non-collagen protein) and non-organic (mineral) materials. Neonates are in a rapid state of growth and their requirements of minerals and proteins are very high. Bones in neonates not only provide structural support and facilitate development but also participate in mineral homeostasis. Disorders of bone and minerals in neonates therefore have significant local and systemic impact. This chapter deals with clinical presentation and management of disorders of bone and mineral metabolism which manifests in the perinatal and neonatal period. Disorders covered in this chapter are:

1. Disorders of mineral metabolism:
   a. Metabolic bone disease of prematurity
   b. Disorders causing neonatal hypocalcemia
   c. Disorders causing neonatal hypercalcemia
   d. Disorders causing neonatal hypomagnesemia
2. Disorders of mineral deposition: hypophosphatasia and generalised arterial calcification of infancy
3. Defects in bone matrix formation: osteogenesis imperfecta
4. Sclerosing bone disorders
5. Skeletal dysplasia: achondroplasia

2. Disorders of mineral metabolism

2.1. Metabolic bone disease of prematurity

Metabolic bone disease of prematurity (MBDP) is a common bone mineral metabolic disorder in neonatal practice, given the increasing numbers of extremely preterm and low birth weight infants now surviving. In utero, maximal bone mineral accretion occurs in the third trimester [1,2]. Clearly, this is interrupted when a neonate is born sufficiently preterm. Due to limitations in calcium and phosphate content of human milk, as well as limitations in enteral mineral absorption, postnatal nutrition can never match the in utero mineral supply that would have occurred during this period. This results in undermineralisation of the preterm skeleton, with typical radiographic appearances of osteopenia and rickets. MBDP is further exacerbated postnatally by other complications of prematurity (bronchopulmonary dysplasia, necrotising enterocolitis), medications (glucocorticoids, loop diuretics, antacids, parenteral nutrition) and reduced physical activity [3,4]. MBDP can result in fragility fractures, typically of the long bones and ribs. The most recent study of this suggested that 34% of extremely low birthweight infants with radiological evidence of MBDP sustained a spontaneous fracture [5]. However, the exact incidence of fragility fractures following MBDP is unknown, partly as they are often found incidentally, even after discharge from neonatal units which can result in safeguarding concerns.

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[*] Corresponding author. Consultant Paediatric Endocrinology, Royal Manchester Children's Hospital, Manchester, M13 9WL, UK.
E-mail addresses: Amish.chinoy@mft.nhs.uk (A. Chinoy), Zulf.mughal@mft.nhs.uk (M.Z. Mughal), Raja.padidela@mft.nhs.uk (R. Padidela).

https://doi.org/10.1016/j.siny.2019.101075

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2.1.1. Prevention

Prevention of MBDP is by the use of milk preparations that contain adequate concentrations of calcium and phosphate, as it is deficiency of these minerals that causes MBDP. Therefore, fortification of breast milk and use of preterm formulas should be standard practice in at-risk groups. Current recommendations from preterm nutritional consensus guidelines from the American Academy of Paediatrics and the European Society for Paediatric Gastroenterology, Hepatology and Nutrition are to provide 120–200 mg/kg/day of calcium and 60–140 mg/kg/day of phosphate in enteral feeds [6,7]. The ratio of enteral calcium to phosphate intake is important, with evidence suggesting that the optimal ratio for absorption and retention of both minerals is 1.5:1 to 1.7:1 on a mg-to-mg basis [6]. For parenteral nutrition, current American and European guidance recommends 1.3–3.0 mmol/kg/day of calcium and 1.0–2.3 mmol/kg/day of phosphate be supplied through parenteral nutrition, at a molar calcium to phosphate ratio of 1.3:1 to 1.7:1 [8, 9].

The importance of vitamin D in mineral absorption specifically in the preterm infant is unclear. Nonetheless, supplementation with vitamin D (cholecalciferol) is recommended in preterm neonates, with the aim of maintaining serum 25-hydroxyvitamin D above 50 nmol/l [6].

In utero, bone formation is stimulated by mechanical loading experienced from movements against the uterine walls, which is removed when the neonate is delivered preterm [10]. Although encouraging results have been demonstrated for physical activity programmes in the short-term, long-term data is lacking [11–13].

2.1.2. Treatment

Despite the above preventative measures, calcium and phosphate mineral deficiency can still occur relative to in utero mineral accretion, resulting in MBDP. The preterm gut absorbs much greater phosphorus than calcium (80–90% versus 50–60% respectively) [14,15]. Therefore, in our experience, calcium deficiency tends to be the commoner aetiology. This is suggested by a secondary elevation in plasma parathyroid hormone (PTH) to maintain normocalcemia. On the other hand, there is no such compensatory mechanism in phosphate deficiency, and therefore the plasma PTH is normal in phosphate deficiency. Importantly, hypophosphatemia is therefore a universal feature of MBDP, regardless of aetiology – in calcium deficiency it occurs due to PTH mediated renal phosphate excretion and in phosphate deficiency it occurs due to reduced phosphate absorption. Therefore, plasma PTH levels will help identify the primary aetiology.

Understanding the primary aetiology and underlying biochemistry is important in guiding appropriate supplementation. In a primarily calcium deficient state, calcium supplementation is required to normalise elevated plasma PTH, thus reversing the resultant bone resorption and hypophosphatemia (Fig. 1) [16]. Daily doses of 0.5–1.5 mmol/kg in 2–4 divided doses is suggested [6]. Care must be taken not to give calcium supplementation with feeds, as it will precipitate with phosphate in the feed. Conversely, in this primarily calcium deficient state, phosphate supplementation will result in binding to ionised calcium, therefore causing a further increase in PTH and in fact exacerbation of MBDP (Fig. 1) [17]. Therefore the common practice of universal phosphate supplementation of all neonates with MBDP [18] is to be discouraged without prior measurement of plasma PTH.

Should a primarily phosphate deficient state be suggested by a low serum phosphate for age and normal plasma PTH level, phosphate supplementation can be initiated (0.5–1.0 mmol/kg/day in 2–3 divided doses). To maintain optimal enteral calcium to phosphate intake ratios (1.5:1 to 1.7:1 on a mg-to-mg basis), concomitant calcium supplementation may also be necessary. Care must be taken to avoid giving the two supplements simultaneously or with meals, to avoid precipitation.

Monitoring of MBDP resolution, and therefore adjustment of supplement dosage, should be biochemical (serum phosphate, alkaline phosphatase (ALP) and plasma PTH) on a weekly or fortnightly basis.

Active vitamin D analogues (alfacalcidol, calcitriol) are often used in neonatal practice to treat MBDP [18]. Presumably, this was because of a perceived immaturity of 25-hydroxylation in the preterm infant. Given that this has been demonstrated not to be the case [19], and with no pathophysiological rationale for their use in this setting, active vitamin D analogues should be avoided for the treatment of MBDP. Exceptions to this include neonates with renal or hepatic failure, where expert advice should be sought.

2.2. Hypocalcemic disorders

Tightly maintaining serum calcium concentration to 8.8–10.4 mg/dl (2.2–2.6 mmol/L) is important for neuronal and muscle function. This is achieved by the calcium sensing receptor (CaSR) in parathyroid cells. Extracellular ionised calcium activates CaSR, resulting in inhibition of PTH production and release. Therefore in hypocalcemia, CaSR is inactivated resulting in PTH secretion. PTH normalises serum calcium concentration in 3 ways: facilitating conversion of 25-hydroxy-vitamin D to its active form (1,25-dihydroxy-vitamin D) which increases calcium absorption in the gut; reducing renal excretion of calcium; and promoting bone resorption thus releasing bone stores of calcium into the bloodstream. Therefore, abnormalities in PTH production or vitamin D metabolism can result in hypocalcemia. As a neonate's calcium and vitamin D supply is dependent on maternal supply in utero, as well as from breast milk, the mother's vitamin D and calcium status will also be important. The features of hypocalcemia are typically those of

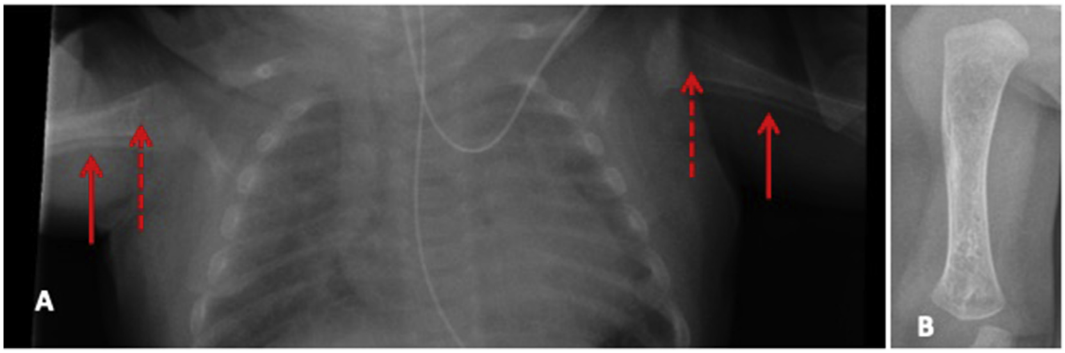

Fig. 1. Radiographs of an infant with metabolic bone disease of prematurity. The infant was born preterm (29-weeks-gestation), suffered from severe chronic lung disease, received multiple courses of dexamethasone and a prolonged period on parenteral nutrition. A: Chest radiograph at 3 months of age, having been on phosphate supplementation and alfacalcidol for several weeks for hypophosphatemia. Note the periosteal elevation (solid arrows) of hyperparathyroidism and metaphyseal rachitic changes (dotted arrows) in both humerii, as well as diffuse osteopenia. B: Right humerus at 6 months of age, following discontinuation of phosphate supplementation and alfacalcidol and 3 months of calcium and vitamin D supplementation. Note the marked improvement in bone mineralisation with resolution of the rachitic appearance and periosteal elevation.

increased neuromuscular excitability – tetany and eventually convulsions. Other features include laryngospasm, stridor and apnoea (particularly in neonates), and cardiac rhythm disturbances. Hypocalcemia is defined as a serum calcium < 8.8 mg/dL (2.2 mmol/L) or an ionised calcium < 4.4 mg/dL (1.1 mmol/L).

In infants, the causes of hypocalcemia include:

- Neonate born to mothers with severe vitamin D deficiency
- Transient hypoparathyroidism: Neonate born to mother with uncontrolled hyperparathyroidism, neonate born premature or term acutely unwell
- Permanent hypoparathyroidism: Transcription factor mutations, Di George syndrome, mutations in the CaSR pathway
- Congenital rickets – Extremely rare cause of neonatal hypocalcemia, with management focussed on calcium and high-dose vitamin D supplementation given the chronic depletion of both in utero [20].
- Vitamin D dependent rickets – Almost exclusively present later in infancy and therefore not discussed further here
- Hypomagnesaemia (discussed elsewhere in this article)

Hypocalcemia in the neonate is usually secondary to severe maternal vitamin D deficiency or hypoparathyroidism, and therefore these will be discussed further below.

2.2.1. Management of acute hypocalcemia

Regardless of aetiology, a neonate that presents with profound hypocalcemia (serum calcium < 7.2 mg/dL (< 1.8 mmol/L) or ionised calcium < 3.6 mg/dL (< 0.9 mmol/L)) or symptomatic with seizures requires urgent correction. Ideally, a blood sample should be collected for measurement of plasma PTH prior to correction of hypocalcemia to allow interpretation of adequacy of PTH response. Urgent correction is usually with a slow intravenous injection of calcium gluconate (0.11–0.46 mmol/kg) over 5–10 min. This should be followed by a continuous intravenous infusion, initially at 0.5 mmol/kg/day, adjusted according to response. Care must be taken with the use of intravenous calcium regarding extravasation injuries, and ideally this should be delivered via a central venous catheter, with conversion to an enteral route as soon as possible.

2.2.2. Hypocalcemia secondary to severe maternal vitamin D deficiency

Maternal vitamin D deficiency does not affect the fetal serum calcium concentration, as calcium is provided directly from the mother without requiring vitamin D [21]. Therefore, at birth these infants are normocalcemic [22]. However, following birth, there is a dependence upon active vitamin D to absorb calcium from the gut. Therefore vitamin D deficiency in the neonate, due to poor maternal supply, will result in hypocalcemia postnatally [22,23]. This is exacerbated in breast-fed infants as vitamin D metabolites do not readily pass into the milk [21]. Following acute management of the hypocalcemia, it is therefore important to check serum calcium, 25-hydroxy-vitamin D and plasma PTH of the mother, as well as serum 25-hydroxy-vitamin D of the neonate. Calcium supplementation and treatments doses of cholecalciferol are required for the neonate, although calcium supplementation can often be discontinued after a few weeks, once the neonate is vitamin D-replete. Thereafter, maintenance doses of cholecalciferol are required. On occasion, hypoparathyroidism or PTH resistance develops in these neonates [24]. The latter is indicated by hyperphosphatemia despite elevated PTH and is presumably in response to chronic hypocalcemia. In this circumstance, active vitamin D analogues (alfacalcidol or calcitriol) may be required for a short duration until the PTH dysfunction resolves and the body is able to convert vitamin D provided through supplementation into active vitamin D.

2.2.3. Hypoparathyroidism

Impaired PTH secretion will result in an inability to maintain normocalcemia. Congenital hypoparathyroidism will usually present during the neonatal period with a low or inappropriately normal plasma PTH despite normocalcemia. Several genetic aetiologies underlie congenital hypoparathyroidism. Describing these is beyond the scope of this article, and has been reviewed elsewhere [25]. Furthermore, transient neonatal hypoparathyroidism can occur in neonates born to mothers with uncontrolled hyperparathyroidism (with fetal hypoparathyroidism in response to hypercalcemia in utero), as well as in the preterm or sick neonate. Regardless of the aetiology of hypoparathyroidism, the treatment concepts remain the same, although the treatment durations will differ.

The conventional management of hypoparathyroidism centres on calcium supplementation (0.5–1.0 mmol/kg/day in 2–3 divided doses) and active vitamin D analogues such as calcitriol (15 ng/kg once daily or in two divided doses) or alfacalcidol (25–50 ng/kg once daily) titrated upwards as necessary. As there is a lack of PTH to promote conversion of vitamin D to its active form, active vitamin D analogues are used in the long-term to maintain normocalcemia by increasing enteral calcium absorption. Oral calcium supplementation may be needed initially until an adequate dosage of active vitamin D analogues is established. However, close monitoring is essential with this conventional management, as the lack of PTH can readily result in hypercalciuria and subsequent nephrocalcinosis, even with normocalcemia. Indeed, thiazide diuretics have been used, even in neonates, to increase renal calcium reabsorption and minimise hypercalciuria while trying to promote calcium retention [26]. Therefore, common practice is to maintain the serum calcium concentration just below the normal range at 8.0–8.8 mg/dL (2.0–2.2 mmol/L), to avoid symptomatic hypocalcemia as well as hypercalciuria [27]. Regular measurement of urinary calcium-creatinine ratios is undertaken in older children to monitor for hypercalciruia, although robust reference ranges in infants are not well-established.

With most hormonal deficiencies, hormone replacement therapies exist. Although forms of recombinant human PTH (rhPTH) do exist, their use in paediatrics has been extremely cautious due to the significantly increased incidence of osteosarcomas in rat studies when supraphysiological doses were used [28,29]. However, such concerns have not arisen in clinical surveillance studies [30]. Two forms of rhPTH exist – rhPTH [1–34], also known as teriparatide, which is the active fragment of PTH; and rhPTH [1–84] which is the full PTH peptide; both are delivered subcutaneously. As rhPTH [1–34] is used almost exclusively so far in the paediatric literature for hypoparathyroidism, this shall be the focus in this review. A randomised 3 year trial of children with hypoparathyroidism demonstrated long-term safety and efficacy of teriparatide compared to conventional treatment [31]. In terms of dosing regimens, a twice daily regime of teriparatide resulted in less variation in serum calcium concentration and lower total dose requirement than a once daily regime, in a cross-over study [32]. More recently, continuous subcutaneous pump delivery of teriparatide has also been shown in children to ameliorate some of the hypercalciuria that is not completely prevented using the twice-daily dosing regimen, as well as a further reduction in total dose requirements and variability in serum calcium concentrations [33]. Specifically within the neonatal literature, use of teriparatide is reported in refractory cases of congenital hypoparathyroidism, with the authors also successfully having utilised teriparatide in such cases [26]. Case reports are also noted of using teriparatide to manage refractory acute hypocalcemia in neonates [34,35]. Given that rat studies have suggested that the development of osteosarcoma with teriparatide is duration-dependent as well as dose-dependent, long-term safety has to be established before recommending this as routine second-line treatment [36]. Furthermore, expense may also limit its use.

Autosomal dominant hypocalcemia (ADH) is a form of hypoparathyroidism that results from activating mutations in the CaSR (ADH type 1), and in GNA11 (ADH type 2) – which encodes for a key mediator of CaSR signalling. These activating mutations result in alteration of the 'set point' for detecting hypocalcemia by the parathyroid gland, resulting in reduced PTH release during hypocalcemia. Management is either with conventional treatment as outlined above, or with teriparatide considered in refractory cases [26]. Calcilytics inhibit CaSR function, and therefore would promote PTH release, potentially correcting the underlying pathophysiology of ADH. In vitro and animal studies have recently examined the potential of calcilytics in ADH, demonstrating an improvement in serum calcium and PTH concentrations, as well as a reduction in urinary calcium excretion [37]. A recent early phase clinical trial in 5 adults with ADH has similarly shown promising results for future research [38].

2.3. Hypercalcemic disorders

Hypercalcemia is rare in neonates and is defined as serum calcium > 10.8 mg/dl (2.7 mmol/l) or ionised calcium > 5.4 mg/dl (1.35 mmol/l). Symptoms of hypercalcemia are non-specific such as irritability, hypotonia, feeding difficulties, failure to thrive, polyuria and seizures. Therefore, diagnosis is invariably confirmed through routine blood tests. The consequences of hypercalcemia are however significant with complications of cardiac arrhythmias, hypertension, nephrocalcinoisis, nephrolithiasis and renal failure.

Management of hypercalcemia is based on aetiology and therefore we have classified neonatal hypercalcemia into the following two categories:

Hypercalcemia with normal or elevated parathyroid hormone.

1. Familial Hypocalciuric Hypercalcemia (FHH) secondary to heterozygous inactivating Ca sensing receptor (CaSR) mutation (*CASR* gene), a benign disorder: Normal PTH.
2. Neonatal Severe Hyperparathyroidism (NSHPT) secondary to homozygous inactivating *CASR* mutation: Elevated PTH.
3. Neonatal Hyperparathyroidism, a variant of FHH when heterozygous inactivating mutation of *CASR* is paternally inherited: Elevated PTH.

Hypercalcemia with suppressed parathyroid hormone.

1. Iatrogenic: Vitamin D or calcium (oral or IV) intoxication
2. Subcutaneous fat necrosis (SFN): History of foetal distress and/or birth asphyxia
3. Hypophosphatasia
4. Williams-Beuren Syndrome
5. Hypercalcemia of infancy: *CYP24A1* mutation
6. Idiopathic hypercalcemia of infancy

2.3.1. Management of hypercalcemia in neonates

Neonates should be assessed for degree of dehydration, presence of subcutaneous fat necrosis and calcification, and fractures.

Investigations for hypercalcemia should include calcium, phosphate, ALP, PTH, 25-hydroxy-vitamin D and urinary excretion of calcium. The above investigations should also be performed on parents as they may have benign FHH which may not yet have been diagnosed. If PTH is elevated and fractures are suspected, radiographs of limbs for features of periosteal elevation, osteopenia and fractures should be performed.

Neonates with FHH are generally asymptomatic and diagnosis is made incidentally when serum calcium is high, usually between 2.7 and 3.5 mmol/l (11–14 mg/dl). PTH concentration is generally within the normal range or marginally elevated with very low urinary excretion of calcium (calcium/creatinine ratio of < 0.5). In FHH high calcium is perceived as normal by CaSR and therefore, misdiagnosis and initiation of a low calcium diet or other interventions to lower calcium can paradoxically worsen the condition with elevation of PTH and ensuing osteopenia, subperiosteal erosions and fractures.

In NSHPT, serum calcium levels are usually > 14 mg/dl (> 3.5 mmol/l) and may be as high as 20–30 mg/dl (5–7.5 mmol/l) with significantly elevated PTH. Neonates present with dehydration, hypotonia, feeding difficulties and respiratory distress. High PTH causes significant demineralisation of the skeleton with radiographs demonstrating periosteal reaction and subperiosteal erosions, osteopenia, bell shaped thoracic deformities and fractures. If untreated, NSHPT is potentially fatal and parathyroidectomy is invariably required. Preoperative management consists of hyperhydration with saline (150–200 ml/kg/day), diuretics and intravenous pamidronate (0.25–0.5 mg/kg). In addition, Cinacalcet, an allosteric CaSR agonist, has been found to be useful in reducing serum calcium preoperatively and in some cases stabilizing serum calcium without the need for parathyroidectomy [39]. Cinacalcet should be used with caution as it can cause fatal hypocalcemia in children.

In neonates with FHH, where mutation in CaSR is paternally inherited, the fetus perceives maternal serum calcium to be low and therefore increases fetal PTH secretion. This causes subperiosteal bone resorption, demineralisation of the skeleton and fractures. Biochemically, neonates present with normal or marginally elevated serum calcium, high PTH and radiographs showing periosteal elevation, osteopenia and fractures. Paradoxically, increasing serum calcium concentration to around 12.0-12.8 mg/dl (3.0-3.2 mmol/l) reduces serum PTH and heals skeletal deformities. Alternatively, cinacalcet could be used to reduce PTH concentration. Neonatal FHH with paternally inherited heterozygous R185Q mutation in *CASR* gene present with severity similar to NSHPT described above and there are reports of successful management with high doses of cinacalcet [40].

Subcutaneous fat necrosis (SFN), also known as transient panniculitis, is seen in neonates following trauma at birth, meconium aspiration and therapeutic cooling for birth asphyxia [41]. A small proportion of these infants develop hypercalcemia. Clinically, neonates present with failure to thrive, vomiting, irritability, fever and firm erythematous nodules or plaques arising from subcutaneous tissue. Serum calcium as high as 20 mg/dl (4.8 mmol/l) have been reported [42]. Inflammatory cells within fat necrosis have increased expression of 1-α hydroxylase activity which produces 1,25-dihydroxy-vitamin D leading to hyperabsorption of dietary calcium.

Treatment consists of hyperhydration, diuretics and low calcium formula feeds (Locasol, Nutricia Ltd, UK and Calcilo XD, Abbott, USA). Prednisolone or methylprednisolone at a dose of 1–2 mg/kg/day allows suppression of 1-α hydroxylase activity and normalises calcium levels, but treatment may be required for up to 5 months [41]. A single dose of pamidronate (0.25–0.5 mg/kg) can normalise serum calcium, following which it can be maintained within normal range by low calcium formula feeds [41,42]. Special feeds will be required to be continued till serum PTH levels normalise suggesting that the condition is resolving. Nephrocalcinosis is present in most infants at the time of diagnosis and does not resolve with treatment, but does not impact on renal function [41].

Vitamin D intoxication associated hypercalcemia is rare in neonates. If inadvertent high dose over the counter cholecalciferol is administered from birth, hypercalcemia presents after 1 month of age [43]. Very high serum concentration of 25-hydroxy-vitamind D is required for producing hypercalcemia. Management consists of discontinuing supplementation and in symptomatic infants hyperhydration, diuretics, low calcium formula feeds and if required intravenous pamidronate.

Williams-Beuren Syndrome, hypercalcemia of infancy secondary to *CYP24A1* mutation and idiopathic hypercalcemia of infancy rarely presents in the neonatal period and treatment is similar to other causes of hypercalcemia where PTH is suppressed.

2.4. Hypomagnesemia

Serum magnesium concentration is regulated by gut absorption (both passively and actively by the transient receptor potential melastatin-6 and -7 (TRPM6 and TRPM7) channels) and renal excretion at the distal convoluted tubule [44]. In neonates, hypomagnesemia may

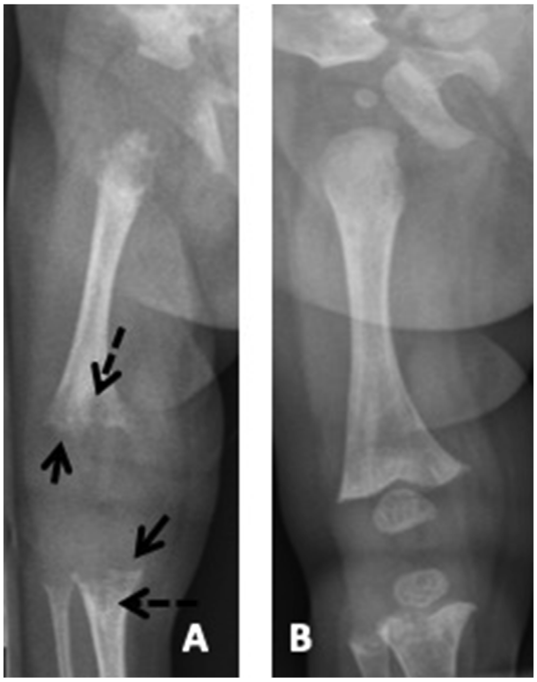

Fig. 2. Skeletal features of hypophosphatasia (HPP) before (A) and 3 months after (B) asfotase alfa treatment in a neonate with the infantile form of HPP. Radiographs of right femur and upper end of tibia and fibula before treatment (A) show undermineralization, severe rickets (bold arrows), absence of epiphysis and characteristic tongue-like metaphyseal lucencies at the end of the long bones (dotted arrows). Post treatment (B) there is significant healing of rickets, improved mineralisation and appearance of epiphysis.

be observed due to maternal hypomagnesemia, malabsorption, birth asphyxia, intrauterine growth restriction and infants born to diabetic mothers [45]. In addition, an autosomal recessive condition called hypomagnesemia with secondary hypocalcemia (HSH) due to mutations in the *TRPM6* gene also present in the neonatal period with seizures [46–48]. The importance of magnesium in calcium regulation is through its complex relationship with PTH (reviewed in Ref. [49]). Significant hypomagnesemia inhibits secretion of PTH in response to hypocalcemia. Therefore *all* neonates presenting with hypocalcemia, particularly those with hypoparathyroidism, must have their serum magnesium concentration assessed and hypomagnesemia treated concurrently with hypocalcemia. This is usually with oral magnesium supplements (magnesium glycerophosphate at a dose of 0.2 mmol/kg 3 times a day), although intravenous magnesium may be required if tolerance is an issue (magnesium sulphate 100 mg/kg 6–12 hourly), particularly if high doses are required as in HSH [46–48]. Prompt recognition and treatment of hypomagnesemia at presentation, particularly in HSH, is important to avoid long-term neurological morbidity [47,48].

3. Disorders of mineral deposition

Bone mineralisation occurs through deposition of inorganic hydroxyapatite crystals (HA) [$Ca_{10}(PO_4)_6(OH)_2$] onto extracellular organic collagen fibres. Mineralisation is initiated in the matrix vesicles of osteoblasts where calcium and inorganic phosphate (Pi) combines to form HA. To promote mineralisation within bones and to inhibit in non-bone tissues, there are mediators and inhibitors of mineralisation. Circulating inorganic pyrophosphate (PPi) inhibits mineralisation while high concentration of Pi promotes mineralisation. A higher ratio of Pi/PPi is therefore required for mineralisation. Within bones, high concentration of alkaline phosphatase (ALP) promotes mineralisation by hydrolysing PPi to Pi thereby increasing ratio of Pi/PPi. ALP is coded by the *ALPL* gene while PPi is generated by ecto-nucleotide pyrophosphatase (ENPP), which is coded by *ENPP1* gene.

Mutations in *ALPL* causes Hypophosphatasia (HPP) where low circulating ALP leads to high circulating PPi, causing undermineralization of skeleton as well as rickets and osteomalacia. In addition, ALP also dephosphorylates pyridoxal 5′-phosphate (PLP) to pyridoxine which crosses the blood brain barrier to produce neurotransmitters and therefore low levels of pyridoxine, in the most severe cases, causes pyridoxine-response seizures. While mutations in *ALPL* causes undermineralization of the skeleton, mutations in *ENPP1* and ATP-binding cassette C subfamily, member 6 (*ABCC6*) causes diagonally opposite disorder, generalised arterial calcification of infancy (GACI). The mechanism by which *ABCC6* mutation causes GACI is not clear; it is hypothesized that ABCC6 generates circulatory factors in liver, which in the physiological state inhibits arterial mineralisation and therefore loss of ABCC6 function causes GACI.

Both HPP and GACI are clinically heterogenous and most severe forms are fatal.

3.1. Hypophosphatasia

Two clinical forms of HPP can present in the neonatal period; the most severe perinatal form presents with significantly undermineralised skeleton during fetal development, while the infantile form presents within 6 months of age.

The perinatal form develops skeletal manifestations during fetal development and may be diagnosed with antenatal ultrasound features of undermineralized skeleton. At birth infants are significantly hypotonic with radiological features of undermineralized skeleton and absence of ossification centres of multiple bones and severe rickets. A benign perinatal form of HPP has been described where the fetus shows USS features of the perinatal form but by birth most skeletal features spontaneously improve. Most neonates with the perinatal form will require some form of respiratory support, with the most severe requiring assisted mechanical ventilation.

The infantile form presents within 6 months of age with severe failure to thrive and the above skeletal features. In both forms, failure of mineralisation leads to hypercalcemia and hyperphosphatemia which causes nephrocalcinosis and renal stones. Paradoxically, infants progress to develop craniosynostosis with raised intracranial pressure.

Biochemical features are characterised by very low ALP, hypercalcemia, hyperphosphatemia and elevated PPi and PLP. Radiological features of HPP are shown in Fig. 2A.

3.1.1. Management of hypophosphatasia

Without treatment, the perinatal form of HPP is fatal while there is 50% mortality in the infantile form. Asfotase alfa (AA), a recombinant enzyme, is now licensed for the management of HPP. In a non-randomised open-label study of 10 infants with perinatal and infantile forms of HPP there was improvement in survival (one died of sepsis), biochemical parameters, skeletal mineralisation and physical and pulmonary function [50]. A follow up study of these infants for 7 years continues to show improvement with survival, skeletal mineralisation and neurodevelopmental outcomes [50].

Multidisciplinary team consisting of metabolic bone disease specialists, intensivists, pulmonologists and physical rehabilitation therapists are required for the management of HPP with AA. Dose is typically 2 mg/kg/dose administered 3 times a week. A higher dose may be required, but expert opinion should be sought for this. Most neonates will require prolonged ventilation and paediatric pulmonologist will be required for planning long-term ventilation. Assisted feeding through nasogastric tube or gastrostomy may be initially required as swallowing is unsafe. Mineralisation of skeleton with AA resolves hypercalcemia and hyperphosphatemia, but initially low calcium formula feeds (Locasol, Nutricia Ltd, UK and Calcilo XD, Abbott, USA) may be required to prevent nephrocalcinosis. Physical rehabilitation helps in fracture prevention, proper positioning and handling, and facilitates physical development.

Following treatment with AA there is normalization of PPi and PLP. Bones undergo mineralisation and rickets shows significant healing (Fig. 2B).

AA has been found to be safe and effective in the management of HPP, however not all features of HPP such as dental phenotype, craniosynostosis and failure to thrive are completely rescued. In addition, long term safety needs to be investigated as suppression of PPi can cause vascular and ectopic mineralisation as is seen in GACI.

3.2. Generalised arterial calcification of infancy (GACI)

Mutations in the *ENPP1* gene causes the clinically heterogenous disorder of GAC, which is characterised by low circulating concentration of PPi, an inhibitor of mineralisation. Mutations in *ABCC6* causes GACI and pseudoxanthoma elasticum (PE). There is phenotypic overlap between these mutations as some patients with *ENPP1* mutation also have PE.

Neonates with GACI develop calcification of media and proliferation of intima of medium and large sized arteries. Age of presentation is bimodal with around half presenting in utero or within the first week of age while around half presenting at a median age of 3 months [51]. There is no genotype-phenotype correlation in GACI.

In early onset presentation, antenatal ultrasonography may demonstrate increased echogenicity of the calcified main arterial vessels as early as 18 weeks of gestational age. Progressive stenosis of the vessels manifests with non-specific symptoms in neonates with irritability, hypertension, cardiomegaly and heart failure, respiratory distress and stroke with death within the first 6 months in most severe cases. In the presence of above symptoms, diagnosis of GACI in the postnatal period is incidental on identification of calcification of vessels on radiographs, ultrasonography or CT scans. Bone biochemistry is essentially normal with low circulating PPi, the measurement of which is available from very few specialist laboratories (Prof Frank Rutsch, Münster University Children's Hospital, Münster, Germany). Hypophosphatemia with hypophosphatemic rickets may develop in surviving infants, the mechanism of which is not known.

3.2.1. Management of GACI

Management of neonates with GACI is multidisciplinary with support from neonatologist, bone disease specialists, cardiologist and nephrologist for the management of heart failure and hypertension respectively. There are no licensed medications for management of GACI. Retrospective studies have demonstrated beneficial effect of bisphosphonates in improving survival [52]. Etidronate, a first-generation bisphosphonate, which is a synthetic analogue of PPi has been most frequently used at a dose is 5–35 mg/day. Newer generation bisphosphonates such as intravenous pamidronate and oral risedronate have also been demonstrated to improve survival. Since manifestations of arterial calcifications can't be potentially reversed, antenatal administration of oral etidronate to pregnant women has been successfully reported [53].

It would be important to note that none of the medications have been subjected to prospective studies or randomised trial and there have been reports of survival with conservative supportive care and mortality in those who have received bisphosphonates, suggesting that there is phenotypic variability and survival of neonates with bisphosphonates may be a reflection of natural history of the disease.

ENPP1-Fc fusion protein has been developed which in animal models of GACI has shown complete clinical response in improving manifestation of arterial calcification [54]. Following demonstration of success in animal models, there is potential for ENPP1-Fc fusion protein to undergo clinical trials in humans for management of GACI.

4. Disorder of bone matrix formation: osteogenesis imperfecta

Osteogenesis imperfecta (OI) refers to a group of conditions of bone fragility secondary to inherited collagen-related connective tissue disorders. Patients present with varying degree of bone fragility; most severe form of this presents with extensive intrauterine fractures and bone deformities and may be lethal. Those with moderate severity may present with multiple long bone and vertebral fractures. Some infants come to attention following investigations for non-accidental injuries because of unexplained fractures, especially in the absence of a family history of OI.

4.1. Classification

OI was first classified by Sillence into four types based on clinical and radiological features. Autosomal dominant (AD) mutations in *COL1A1* and *COL1A2* genes were found to cause these OI's. Since 2005 the nosology of OI has expanded with many more AD and autosomal recessive (AR) OI now described. Recently a new way of classifying OI has been attempted based on altered intracellular or extracellular metabolic pathways which has been summarised below (with associated gene defect); those marked with an asterix are clinically severe to moderately-severe and are likely to present in the neonatal period or early infancy [55].

1. Defects in collagen synthesis, structure, or processing (group A)

   AD: Types I, II*, III* and IV* (*COLA1* and *COL1A2*).
   AR: Type XIII (*BMP1*).

2. Defects in collagen modification (group B)

   AR: Type VII* (CRTAP), Type VIII* (*LEPRE1/P3H1*), Type IX* (PPIB), Type XIV* (*TMEM38B*).

3. Defects in collagen folding and cross-linking (group C)

   AR: Type X* (SERPINH1), Type XI* (FKBP10), Unclassified* (P*LOD2*).

4. Defects in bone mineralisation (group D)

   AD: Type V (*IFITM5*).
   AR: Type VI* (SERPINF1).

5. Defects in osteoblast development with collagen insufficiency (group E)

   AR: Type XII* (SP7), Type XV* (WNT1), Type XVI* (CREB3L1).

4.2. Diagnosis of OI in neonates

In neonates presenting with explained or unexplained fractures, diagnosis of OI should be considered. Diagnosis in neonates is initially made on clinical features, family history (30% are due to de novo mutations) and radiological features of OI. Most severe cases (types II, III, VII, VIII, IX and X) are likely to present with history of antenatal scans showing features of hypomineralization, intrauterine fractures and/or bone deformities as early as 16 weeks of gestational age. In the absence of family history, antenatal scan features are difficult to differentiate from achondrogenesis type I, campomelic and thanatophoric dysplasia [56]. At birth, clinical features of OI in these infants may include, blue sclera, relative macrocephaly, soft calvaria with flattening of the occiput, narrow thoracic cage with respiratory distress requiring supplementary oxygen or mechanic ventilation in most severe cases. Limbs are short and deformed with paucity of movement because of

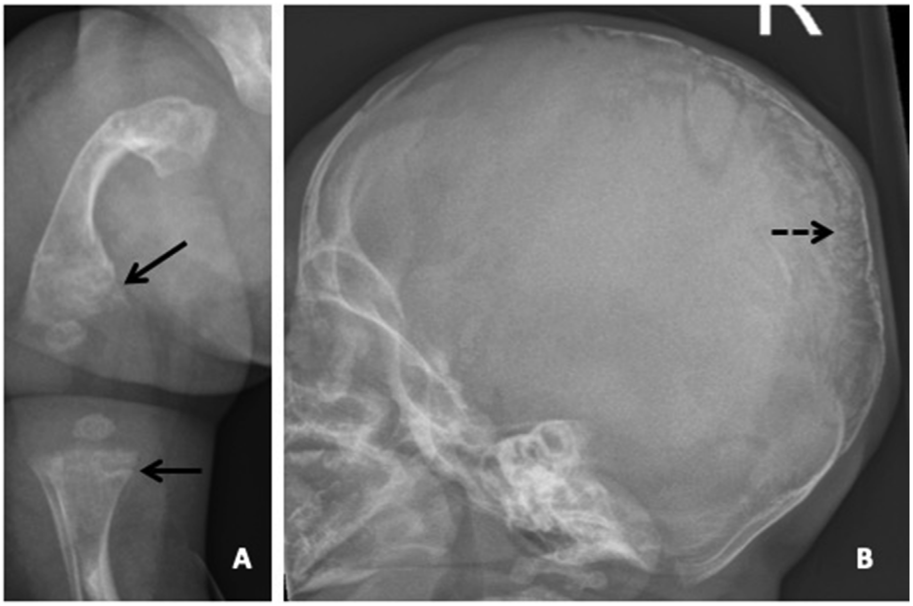

Fig. 3. Radiographs of severe osteogenesis imperfecta (OI) at birth. Right femur (A) is short and. bowed secondary to intrauterine fractures. Metaphyses of femur and tibia are broad with abnormal calcifications (bold arrows). Skull vault (B) shows reduced mineralisation with multiple Wormian bones (dotted arrow).

hypotonia (muscle weakness is a feature of OI) and pain from fractures. Besides skeletal changes, some neonates may have umbilical and inguinal hernias.

Neonates with moderate severity may only present with long bone and/or vertebral fractures on radiographs, therefore in neonates presenting with fractures following minor trauma or unexplained fractures, besides investigating for inflicted injuries, the possibility of OI should be considered. Fig. 3 demonstrates some of the typical radiological features of severe OI.

4.3. Management of OI in neonates

About 90% of neonates with severe types of OI and ~50% with moderately severe OI sustain fractures during delivery. Mode of delivery does not influence the risk of fractures and therefore decision for caesarean mode of delivery should be based on maternal and fetal indications only [57].

Multidisciplinary team, consisting of neonatologist, metabolic bone disease specialists, orthopaedics and physical therapists are required for management of severe and moderately severe OI in neonates. There is no cure for OI and management is geared at supportive neonatal care, pain relief, increasing bone mineralisation by intravenous bisphosphonates (IVB) and physical rehabilitation for proper positioning, handling and transport. Unfortunately, there are no prospective longitudinal studies or controlled trials to generate evidence for the above approach. Recommendations for management is based on collective experience of authors and other experts with experience of management of severe OI in neonates.

Neonates with severe OI are initially best nursed in the neonatal unit where respiratory support and feeding requirements of the neonate can be addressed. Very rarely, assisted mechanical ventilation may be required which, if long term, will necessitate insertion of tracheostomy. Most have swallowing difficulties and would require nasogastric or gastrostomy feeding. Central venous catheter is required for management of fluids and for administration of bisphosphonates; this avoids difficult cannulation attempts which increases risk of fractures.

4.3.1. Intravenous bisphosphonates (IVB) in neonates

Cyclic IVB (pamidronate or zoledronic acid) is now routinely used for management of severe OI in neonates. OI is a high bone turnover condition where osteoclasts continue to remove abnormally formed bones and therefore reducing bone mineral content and density. IVB inhibits osteoclasts and therefore increases bone mineral content and density, however it is unable to change the quality of bone formation. Treatment has been reported to be safe, however evidence for effectiveness in neonates is lacking barring few case series demonstrating reduction in the risk of long-bone and vertebral fractures and improvement in bone mineral density [58–60].

There are no consensus on dose and frequency of administration of IVB; a comparative study of 6 or 12 mg/kg/year demonstrated higher dose led to higher increase in spinal bone mass while safety profile and resolution of vertebral deformities were similar [60]. Typically, pamidronate infusion is given over 3 h on 2–3 consecutive days and this cycle is repeated once every 2–3 months. It is advisable to commence on more frequent, smaller dose cycles, before building up to 1 mg/kg/day on 2 consecutive days every 2 months. In older children the first dose of IVB causes flu like symptoms with subsequent infusions well tolerated. In neonates, there is potential for deterioration of respiratory function and therefore this needs to be monitored, preferably in a neonatal intensive care unit [61].

Zoledronic acid is more potent and therefore requires single infusion over 30 min once every 3–6 months at a dose of 0.1 mg/kg/year. Even though some centres have been using this (personal communications), there are no published reports of safety and efficacy in neonates or early infancy.

IVB are nephrotoxic and therefore contraindicated in renal failure. Inhibition of osteoclasts can cause hypocalcemia, especially in the presence of vitamin D and dietary calcium deficiency. Therefore, it is important to ensure that neonates are vitamin D sufficient (serum 25-hydroxy-vitamin D $>$ 50 nmol/l) and have adequate dietary intake of calcium.

4.3.2. Physical rehabilitation

Physical therapy and occupational therapy have now become standard of care for multidisciplinary management of OI in neonates. A recent consensus statement has recognised lack of evidence of interventions, but expert opinion strongly supports physical rehabilitation [62].

Goals of therapy are to facilitate routine care mainly around change of diaper and dressing, feeding, bathing, handling and cuddling.

Neonates are prone to develop platybasia, which increases risk of basilar impression and invagination. Using towel rolls, regular positioning in supine, prone and side lying can facilitate in maintaining head shape. Therapists provide advice on safe handling and positioning to prevent risks of fracture such as using wide hands, slow movements, avoiding picking around ribs, and twisting and turning of limbs. Despite this, neonates are prone to fracture and if fracture occurs, therapists provide advice on positioning for pain relief and also rehabilitation to prevent secondary effects of fractures. While orthopaedic team is involved in MDT care, it is unusual for neonates to require intervention for healing of fractures. Infants are at a high risk of developing spinal fractures and advice is to nurse in flat position to prevent excessive loading on fragile vertebral bodies. This would also require special reclinable car seat, push chair, highchair and bed for maintaining flat position and slow loading of vertebrae.

4.4. *Ethics of treatment and palliative care*

Most neonates with severe OI, especially some type III may survive but have significant deformities, developmental delay, neurological complication and poor quality of life. If prolonged ventilation is required, ethics of continuing treatment should be considered and discussed with the local ethics committee and families. A palliative care team would be required, if end of life care is planned.

4.5. *Future perspectives in the management of severe OI*

Biological agents anabolic to bones such as parathyroid hormone, growth hormone, denosumab and anti-sclerostin antibodies have potential of increasing bone mineral content and density but have not been investigated in children.

Osteoblasts, which form collagen fibres, are derived from mesenchymal stem cells (MSC); fetal infusion of allogenic human first-trimester liver-derived MSCs has shown significant improvement in the phenotype of severe type III and IV OI, however postnatal booster from the same allogenic source was required to maintain its effectiveness [63]. A European multicentre phase I/II study, BOOSTB4 (NCT03706482), with a schedule for prenatal and postnatal infusions is currently underway [63].

5. Sclerosing bone disorders

This group consists of rare heterogenous disorders which according to the latest nosology of genetic skeletal disorders have been classified into following two groups [64]. Those marked with an asterix are lethal in the intrauterine or perinatal period and therefore management of these are not described in this section:

1. Neonatal osteosclerotic dysplasias
   a. Blomstrand dysplasia*: AR mutation in *PTHR1*
   b. Desmosterolosis*: AR mutation in *DHCR24*
   c. Caffey disease: AD mutation in *COL1A1*
   d. Caffey dysplasia*: AR mutation in *COL1A1*
   e. Raine dysplasia: AR mutation in *FAM20C*
   f. Dysplastic cortical hyperostosis, Kozlowski–Tsuruta type*: AR mutation?
   g. Dysplastic cortical hyperostosis, Al-Gazali type*: AR mutation?
2. Osteopetrosis and related disorders
   a. Osteopetrosis, osteoclast rich, severe neonatal or infantile forms: AR mutations, *TCIRG1, CLCN7, SNX10*
   b. Osteopetrosis, osteoclast rich, severe infantile form with nervous system involvement: AR mutation *OSTM1*
   c. Osteopetrosis, osteoclast-poor, infantile form, with immunoglobulin deficiency (OPTB7): AR mutation TNFRSF11A
   d. Pycnodysostosis: AR mutation *CTSK*

5.1. *Management of sclerosing disorders*

Caffey's disease is a form of infantile cortical hyperostosis which presents in the first 6 months of age with fever, irritability and localised hyperostosis secondary to subperiosteal new bone formation. A specific heterozygous mutation in *COL1A1* gene has been found to be associated with Caffey's disease (p.Arg1014Cys) [65]. Lesions are most commonly seen in the mandible, clavicle, ulna, scapula, tibia and ribs. Lesions spontaneously regress by 2 years of age and management is conservative with anti-inflammatories such as indomethacin or naproxen and a short course of corticosteroids. Raine dysplasia is caused by AR mutation in *FAM20C* which is involved in the pathway of bone mineralisation. It is mostly fatal during the neonatal period, but some survive into childhood and adults.

Osteopetrosis is caused by impaired bone resorption by osteoclasts. Clinical symptoms of severe AR manifest within first 3 months of age. Neonates may present with hypocalcemia secondary to failure of osteoclasts to mobilize calcium from bones, cranial nerve entrapment from osteosclerotic bones can lead to feeding difficulties (bulbar nerve), blindness and deafness. Neonates can also develop respiratory distress secondary to thoracic wall deformities and choanal stenosis, anaemia and thrombocytopenia from obliteration of medullary cavity of bones and hepatosplenomegaly from extramedullary haematopoiesis. Hypocalcemia is managed by oral calcium and vitamin D supplements; rarely intravenous calcium may be required for a short duration of time.

Since osteoclasts are derived from the myeloid cell line, hematopoietic stem cell transplantation (HSCT) is effective in some forms of osteopetrosis, especially the osteoclast rich forms. Osteopetrosis with mutation in *OTSM1* and some with *CLCN7* develop neurodegenerative features, which does not respond to HSCT and therefore HSCT in not attempted on them. HSCT after 10 months of age is rarely successful because of graft rejection and autologous reconstitution. In addition, donor selection, method of conditioning and management of comorbidities influence 5-years disease-free survival [66].

Pycnodysostosis is rarely diagnosed in the neonatal period except when there is strong family history. Osteosclerosis similar to osteopetrosis is seen on radiographs and it is therefore not uncommon to misdiagnose. Acroosteolysis of distal phalanges is characteristically present in pycnodystosis, which helps to differentiate it from osteopetrosis in the neonatal period. With age other characteristic features of pycnodysostosis develop, such as characteristic facial features, growth failure, raised intracranial pressure despite open fontanelle and obstructive upper airway sleep apnoea. In severe cases, support for feeding and breathing may be required during neonatal period.

6. Skeletal dysplasia

Skeletal dysplasia refers to a group of several hundred known heritable conditions affecting the growth and development of bone and cartilage. Clearly, description of each of these individually is beyond the scope of this review, but we shall discuss general neonatal management principles before considering specific management of the commonest non-lethal skeletal dysplasia – achondroplasia.

6.1. *General management principles*

A skeletal dysplasia may have been suspected antenatally, based on family history or ultrasound imaging, with prenatal genetic testing also available. Generally, the earlier the abnormalities are detected on ultrasound, the more severe the skeletal dysplasia is likely to be [67]. Identification of a skeletal dysplasia antenatally is beneficial, as it allows for counselling of parents regarding prognosis, with management plans regarding delivery made in advance. However, many skeletal dysplasias are only suspected following birth. Diagnosis should be based on a thorough clinical assessment, radiological features and genetic testing.

Often the most pressing feature when an infant is born with a severe skeletal dysplasia (e.g. asphyxiating thoracic dystrophy – Jeune syndrome) is respiratory compromise. This is due to rib cage abnormalities that affect the size of the thoracic cage resulting in pulmonary hypoplasia [68]. Furthermore, airway anomalies are also commoner in infants with skeletal dysplasia. Such infants may require continuous positive airway pressure or ventilation via an endotracheal tube [68]. If prolonged ventilation is likely to be required, then a tracheostomy would need consideration.

Parental counselling is important, especially following a specific diagnosis when prognosis can be considered. If a lethal skeletal dysplasia is suspected or diagnosed, then discussions regarding supportive and palliative care will be necessary [67].

6.2. Achondroplasia

Achondroplasia is caused by an activating mutation in the *FGFR3* gene, encoding fibroblast growth factor 3. Unexpected infant death occurs in 2–5% of those not regularly monitored, due to cervicomedullary compression from a narrow foramen magnum resulting in central apnoea [69,70]. Current recommendations suggest baseline neuroimaging and polysomnography in all children diagnosed with achondroplasia, with neurosurgical referral for decompression considered if significant [71–73]. Sleep apnoea can also be obstructive in nature in infants with achondroplasia, due to adenotonsillar hypertrophy, which will require relevant intervention by otolaryngology intervention. Hydrocephalus is commoner in achondroplasia, particularly in the first 2 years of life, and requires monitoring by regular measurement of occipitofrontal circumference [72,74]. Kyphoscoliosis is common and usually secondary to unsupported sitting prior to development of adequate truncal strength [75]. Therefore, an occupational therapist is an important part of the multi-disciplinary team, providing advice regarding appropriate seating and carriers. However, sometimes back bracing is required, and rarely surgical intervention [74]. Growth parameters should be regularly monitored, with achondroplasia-specific growth charts used [72]. However, there are no treatments to augment growth, with insufficient data to support the use of recombinant growth hormone [76].

The above management of achondroplasia in the neonate is focussed on monitoring for the important complications. However, recent research has looked into addressing the underlying pathophysiology. Vosoritide is a C-natriuretic peptide (CNP) analog, currently undergoing clinical trials. CNP inhibits the over-activation of the Ras-ERK pathway seen with activation of FGFR3. The Ras-ERK pathway inhibits proliferation of chondrocytes, and therefore vosotritide aims to remove this inhibition. Indeed, a recent phase 2 study has demonstrated safety of vosoritide and a sustained dose-dependent increase in growth velocity in children with achondroplasia [77]. Clearly, we are still some way from using this medication within the neonatal setting.

Declaration of competing interest

AC: no conflict of interest. ZM: consultancy, honoraria and research grants from Alexion (ENB–010–10 study), UK. RP: consultancy, honoraria and research grants from Alexion (ENB–010–10 study), UK.

Practice points

- Metabolic bone disease of prematurity: decision on calcium or phosphate replacement therapy should be based on measurement of serum parathyroid hormone (PTH), alkaline phosphatase, calcium and phosphate. In calcium deficiency state PTH is elevated while in phosphate deficiency it is normal.
- Hypoparathyroidism: Serum calcium concentration should be maintained marginally below the normal range to prevent hypercalciuria and nephrocalcinosis.
- Hypercalcemia with normal or elevated PTH: low calcium diet should be avoided as it can further increase in PTH with subperiosteal bone resorption and fractures.
- Hypophosphatasia: enzyme replacement therapy with asfotase alfa improves survival and prevents life threatening complications.
- Osteogenesis imperfecta: besides medical management with intravenous bisphosphates, physical rehabilitation plays crucial role in preventing complications and improving neuromuscular development.
- Osteopetrosis: prompt neonatal diagnosis and management with hematopoietic stem cell transplantation in osteoclast rich forms improves survival and prevents complications.

Research directions

- Safety and efficacy of rhPTH [1–84], full PTH peptide, in the management of severe hypoparathyroidism in children.
- Safety and efficacy of calcilytics in hypocalcemia secondary to activating mutations in the CaSR (ADH type 1)
- Safety and efficacy of ENPP1-Fc fusion protein in generalised arterial calcification of infancy.
- Safety and efficacy of mesenchymal stem cells transplant and new biological agents for stimulating normal bone formation in osteogenesis imperfecta.

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